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NAC Transcription Factor ORE1 and Senescence-Induced *BIFUNCTIONAL NUCLEASE1* (*BFN1*) Constitute a Regulatory Cascade in *Arabidopsis*

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Running title: ORE1 - *BFN1* regulatory cascade

Summary:

The NAC transcription factor ORE1 is a key regulator of senescence in *Arabidopsis thaliana*. Here, we demonstrate that senescence-induced and cell death-associated *BIFUNCTIONAL NULCEASE1* (*BFN1*) is a direct downstream target of ORE1, revealing a previously unknown regulatory cascade.

Key words: *Arabidopsis thaliana*; senescence; transcription factor; *ORE1*; *BFN1*; promoter

ABSTRACT

Senescence is a highly regulated process that involves the action of a large number of transcription factors. The NAC transcription factor ORE1 (ANAC092) has recently been shown to play a critical role in positively controlling senescence in *Arabidopsis thaliana*, however, no direct target gene through which it exerts its molecular function has been identified previously. Here, we report that *BIFUNCTIONAL NUCLEASE1* (*BFN1*), a well-known senescence-enhanced gene, is directly regulated by ORE1. We detected elevated expression of *BFN1* already 2 hours after induction of *ORE1* in estradiol-inducible *ORE1* overexpression lines and 6 hours after transfection of *Arabidopsis* mesophyll cell protoplasts with a *35S:ORE1* construct. *ORE1* and *BFN1* expression patterns largely overlap, as shown by promoter – reporter gene (*GUS*) fusions, while *BFN1* expression in senescent leaves and the abscission zones of maturing flower organs was virtually absent in *ore1* mutant background. *In vitro* binding site assays revealed a bipartite ORE1 binding site, similar to the one of ORS1, a paralog of ORE1. A bipartite ORE1 binding site was identified in the *BFN1* promoter; mutating the *cis* element within the context of the full-length *BFN1* promoter drastically reduced ORE1-mediated transactivation capacity in transiently transfected *Arabidopsis* mesophyll cell protoplasts. Furthermore, chromatin-immunoprecipitation (ChIP) demonstrates *in vivo* binding of ORE1 to the *BFN1* promoter. We also demonstrate binding of ORE1 *in vivo* to the promoters of two other senescence-associated genes, i.e. *SAG29*/*SWEET15* and *SINA1*, supporting the central role of ORE1 during senescence.

INTRODUCTION

Senescence is a highly regulated process that involves the action of a large number of transcription factors (TFs), and in particular the NAC (for NAM, ATAF1, 2 and CUC2) and WRKY families are enriched for senescence-regulated TFs in many plant species (Guo et al., 2004; Buchanan-Wollaston et al., 2005; Gregersen and Holm, 2007; Balazadeh et al., 2008; Breeze et al., 2011). Recently, several of the more than 20 senescence-upregulated NAC TF genes in *Arabidopsis thaliana* have been shown to regulate senescence, including besides others *AtNAP* (also called *ANAC029*; Guo and Gan, 2006), *ORESARA1* (*ORE1*, *ANAC092, AtNAC2*; Kim et al., 2009; Balazadeh et al., 2010) and *ORESARA1 SISTER1* (*ORS1, ANAC059*; Balazadeh et al., 2011) which all promote senescence. The NAC factor *VASCULAR-RELATED NAC-DOMAIN (VND) INTERACTING2* (*VNI2, ANAC083*) has been reported to integrate ABA signalling and leaf senescence (Yang et al., 2011). Recently, we showed that *JUNGBRUNNEN1* (*JUB1; ANAC042*) negatively regulates senescence, most likely by affecting cellular H_2O_2 homeostasis (Wu et al., 2012).

The probably best characterized senescence regulatory NAC TF is ORE1 that not only plays a key role in the regulation of leaf senescence but also has a function in programmed cell death (PCD). Mutants lacking functional ORE1 protein are delayed in senescence, whereas *ORE1* overexpressors progress faster towards senescence (Kim et al., 2009; Balazadeh et al., 2010). *ORE1* transcript abundance is controlled by at least two molecular mechanisms. First, promoter-reporter (β-glucuronidase, GUS) studies have shown low *ORE1* promoter activity in young, non-senescing leaves, but high activity in senescing leaf parts. Upstream TFs binding to the *ORE1* promoter and regulating its activity have not been reported yet. A second mode of action to control *ORE1* transcript abundance involves *micro-RNA164* (*miR164*), which interacts with *ORE1* mRNA to trigger its degradation. Kim et al. (2009) suggested a trifurcate feed-forward regulatory pathway involving ORE1, *miR164* and EIN2 (ETHYLENE INSENSITIVE2) ensuring a highly robust regulation of leaf senescence and aging-induced cell death. *EIN2* encodes a central protein of ethylene signalling located in the ER (endoplasmic reticulum) membrane (Alonso et al., 1999; Bisson et al., 2009). EIN2 negatively affects *miR164* expression in an age-dependent manner and through this allows *ORE1* mRNA to accumulate, thus acting as a positive control element.

Despite clear evidence for a role of ORE1 in regulating leaf senescence, knowledge about the downstream gene network through which ORE1 exerts its senescence control function is limited. We have previously described genes the expression of which is rapidly induced by

ORE1 activation thus representing potential direct targets of ORE1 (Balazadeh et al., 2010). In addition, bioinformatics analyses and network modelling based on high-resolution time-course profiles of gene expression during leaf development predicted genes whose expression is positively influenced by ORE1 (Breeze et al., 2011). However, a direct target gene of ORE1 has not been demonstrated yet. In the current study, we identified the preferred binding sequence of ORE1 and showed that it directly activates the *BIFUNCTIONAL NUCLEASE1* (*BFN1*) promoter which harbours the element. *BFN1*, which is among the genes that are rapidly and positively regulated by ORE1, encodes a type I nuclease; it shares high amino acid sequence similarity to DSA6 nuclease which is associated with petal senescence in daylily (Panavas et al., 1999), and to ZEN1 nuclease, which is associated with PCD in *Zinnia elegans* (Ito and Fukuda, 2002). *BFN1* expression is known to be specifically enhanced during leaf and stem senescence as well as in the floral abscission zone and during developmental PCD (Perez-Amador et al., 2000; Wagstaff et al., 2009; Farage-Barhom et al., 2011). During late stages of senescence, BFN1 (fused to green fluorescence protein) was found to localize together with fragmented nuclei in membrane-coated vesicles, suggesting a role for BFN1 in regulated nucleic acid breakdown that occurs during senescence and PCD (Farage-Barhom et al., 2011). However, plants lacking functional *BFN1* do not show a detectable change in senescence, most likely due to the fact that multiple genes (e.g. those collectively regulated by ORE1) need to act in concert for the exertion of the full senescence syndrome.

In the present study, we demonstrate that ORE1 specifically activates the *BFN1* promoter and physically interacts with a 40-bp fragment of the *BFN1* promoter containing the ORE1 binding site. Moreover, we show that senescence-enhanced expression of *BFN1* is abolished in *ore1* mutants indicating ORE1 as a major regulator of *BFN1* expression during senescence.

RESULTS

BFN1 Expression is Rapidly induced by ORE1

In our previous studies, we identified genes responding to enhanced *ORE1/ANAC092* expression. More specifically, we have shown that expression of 170 genes significantly increased within 5 h of estradiol (EST) treatment in transgenic lines expressing *ORE1* under the control of an EST-inducible promoter (*ORE1-IOE* plants; Balazadeh et al., 2010). To identify genes more rapidly responding to elevated *ORE1* expression we here repeated the previous experiment, but shortened the EST induction time to 2 h (*ORE1-IOE-2h* dataset).

Furthermore, to exclude potential misinterpretation due to EST treatment we also included an experiment where we transiently expressed a *35S:ORE1* construct in *Arabidopsis* mesophyll cell protoplasts and extracted RNA 6 h after the transfection (*35S:ORE1-6h* dataset); for details see Experimental Procedures. Our data revealed that expression of 831 genes was differentially expressed (two-fold cut-off) upon transient overexpression of *ORE1* in mesophyll cell protoplasts, 643 of which were upregulated and 188 were downregulated relative to Col-0 control protoplasts (**Supplemental Table 1**). Moreover, expression of only 78 genes was differentially expressed (two-fold cut-off) in the *ORE1-IOE-2h* dataset, of which 54 genes were up- and 24 genes were downregulated (**Supplemental Table 2**).

We compared all three transcriptome data sets and identified 17 genes that were commonly upregulated upon elevated *ORE1* expression in stably transformed plants and transiently transfected cells (**Figure 1A and B**), suggesting they are direct downstream targets of ORE1. Among them are genes encoding proteins involved in sugar transport (*SAG29*/*SWEET15*; *At5g13170*), protein degradation (*At3g45010*, serine carboxypeptidase-like 48), protein ubiquitination (*At3g13672*, *SINA1*, a RING E3 ligase), nucleic acid degradation (*At1g11190*, *BIFUNCTIONAL NUCLEASE1*; and *At1g26820, RIBONUCLEASE3*, *RNS3*), and genes encoding protein kinases such as CIPK17 (*At1g48260*) and a cystein-rich receptor-like kinase (*At4g04490*) (**Supplemental Table 3**). Fourteen of the 17 ORE1-regulated transcripts were previously shown to be upregulated during age-dependent senescence (Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Balazadeh et al., 2008; Breeze et al., 2011; Parlitz et al., 2011), and the transcript abundance of 12 genes was reported to be enhanced during long-term salinity (4 days NaCl treatment of 28 day-old plants; for sample description see Balazadeh et al., 2010) (**Supplemental Table 3**). Among the putative ORE1 target genes was *BIFUNCTIONAL NUCLEASE1* (*BFN1*) which encodes a senescence-associated type I nuclease. *BFN1* expression was enhanced by around 4-fold already 2 h after EST treatment in *ORE1-IOE* lines. Its expression further increased to ~40-fold within 5 h of EST treatment. A very strong activation of *BFN1* expression (~1,000-fold) was observed in the *35S:ORE1-6h* protoplast dataset **(Figure 1C)**.

Overlapping Patterns of Transcriptional Activities of *ORE1* and *BFN1* Promoters

The elevated expression of *BFN1* in the cells and transgenic plants overexpressing ORE1 indicated that the NAC TF acts as an upstream positive regulator of *BFN1*. We therefore analysed the extent of co-expression of both genes in *Arabidopsis* plants. Gene expression

profiling data revealed induction of *ORE1* and *BFN1* during age-dependent senescence (Buchanan et al., 2005; Balazadeh et al., 2010; Breeze et al., 2011) and during dark- and salt-induced senescence (Buchanan et al., 2005; our unpublished data). Moreover, expression of both genes is highly induced upon 180 min ABA treatment of 7-day-old seedlings (Arabidopsis Hormone Database; http://ahd.cbi.pku.edu.cn) and significantly reduced in the *ein2* mutant, that lacks a major component of the ethylene signalling pathway (Buchanan et al., 2005). Thus, both genes appear to be under control of the same hormonal signals.

Tissue-specific expression patterns of *BFN1* and *ORE1* were reported earlier using transgenic *Arabidopsis* lines expressing β-glucuronidase (*GUS)* reporter from the 2.3 kb *BFN1* promoter (Farage-Barhom et al., 2008) or the 1.5 kb *ORE1* promoter (Balazadeh et al., 2010), respectively. To investigate the extent of overlapping promoter activities during senescence, we grew the promoter-GUS lines side by side and performed a comparative analysis of gene expression. In general, expression patterns controlled by the *BFN1* and *ORE1* promoters were highly similar in leaves and floral organs at different developmental stages (**Figure 2**). GUS activity was detected in the margins and tips of the oldest leaves, while expression was largely absent in young leaves, consistent with the function of both genes during senescence. GUS activity was also relatively prominent in the stigma, mature anthers, sepals and petals of older/fully opened flowers (flower stages 13 or 14, as classified by Ferrandiz et al., 1999), but not immature flowers. Expression in mature siliques was detectable in abscission zones and in the distal portion of the valve margins for both, $Prom_{ORE1}$*:GUS* and $Prom_{BFN1}$*:GUS* lines (**Figure 2C, H, E and J**). Thus, our data demonstrate largely overlapping expression patterns of the NAC transcription factor gene *ORE1* and its putative downstream target *BFN1*.

Senescence-Specific Expression of *BFN1* Is ORE1-Dependent

In order to test whether the senescence-induced expression of *BFN1* is dependent on the presence of functional ORE1, we tested *BFN1* expression in the *ore1-2* mutant background (Kim et al., 2009), using plants of different developmental stages; plants were grown in soil and leaves were harvested from plants 12, 16, 20, 24, 28, 32 and 36 days after sowing. As shown in **Figure 3**, *BFN1* expression strongly increased with age in wild-type plants; expression was ~64-fold higher in leaves of 28-day-old plants compared to leaves of 24-day-old plants. Expression of *BFN1* remained high and increased further at later time points (in 32- and 36-day-old plants) consistent with previous observations that *BFN1* is a senescence-associated gene (Perez-Amador et al., 2000; Wagstaff et al., 2009; Farage-Barhom et al.,

2011). In contrast, the age-dependent increase of *BFN1* transcript abundance was completely abolished in leaves of the *ore1-2* mutant (**Figure 3A**). These data indicate that senescence-associated expression of *BFN1* is mainly, if not exclusively, regulated by ORE1. Considering the negative regulation of *ORE1* by *miR164* during leaf aging, we next tested *BFN1* transcript level in the *mir164abc* mutant (Kim et al., 2009), at different leaf developmental stages (as described above). Notably, *BFN1* expression started to increase about 8 days earlier in the *mir164abc* mutant than wild-type plants (**Figure 3A**), indicating that *BFN1* expression is under *miR164* control, through its effect on *ORE1* expression.

To further confirm ORE1-dependent *BFN1* expression during senescence, we transformed the *anac092-1* mutant (Balazadeh et al., 2010) with the *Prom-$_{BFN1}$:GUS* construct (Farage-Barhom et al., 2008). To minimise a potential position effect of transgene integration site on *BFN1* promoter activity, we analysed more than 50 independent transgenic *anac092-1*-transformed lines. The presence of the *GUS* gene was confirmed by PCR on genomic DNA. Histochemical staining revealed very low and in most cases undetectable *BFN1* promoter activity in the *anac092-1* transformants, while strong GUS activity was observed in the majority of the Col-0 plants transformed with the *Prom-$_{BFN1}$:GUS* construct (**Figure 3C-E**). Additionally, we quantitatively determined *BFN1* promoter activity by 4-methylumbelliferyl-β-D-glucuronide (4-MUG) assay in young, green leaves (almost fully expanded, of 20-day-old plants) and senescent leaves (20 - 30% yellow leaf area; 40-day-old plants) of *Prom-$_{BFN1}$:GUS* transgenic lines. As shown in **Figure 3C-E**, reporter gene activity was significantly reduced in *Prom$_{BFN1}$:GUS/anac092-1* lines compared with *Prom$_{BFN1}$:GUS*/Col-0 plants. The reduction of GUS activity was particularly pronounced in senescent leaves and the flower abscission zones. These data confirm that ORE1 plays a central role as an upstream transcriptional regulator of *BFN1* expression in *Arabidopsis*.

ORE1 Binding Site

Knowledge about binding sites of transcription factors facilitates the identification of direct target genes within a group of genes early responding after induction of the TF. Originally, Olsen et al. (2005) reported the 9-mer sequence T[TAG][GA]CGT[GA][TCA][TAG] as the preferred binding site of ORE1/ANAC092. Binding sites were also reported for other NAC factors and found to be relatively long, generally consisting of two consensus motifs separated by a spacer of 5 -7 bp, the length of which affects binding affinity (Xue et al., 2005; Zhong et al., 2010; Balazadeh et al., 2011; Wu et al., 2012). Previously, we have reported

[AG]CGT[AG](4-5n)[AG][CT]ACGCAA as the consensus bipartite binding site of the ORS1/ANAC059 transcription factor (Balazadeh et al., 2011); *ORS1* and *ORE1* are phylogenetically closely related genes, representing paralogs (Ooka et al., 2003). Within their NAM domains both proteins share an overall amino acid identity of 94%; sequence identity amounts to around 41% in the C-terminal part of the proteins. Considering the high sequence similarity of the NAM domains of the two NAC TFs, we hypothesized that ORE1 binding specificity resembles that of ORS1. We therefore tested the binding affinity of ORE1 to the known ORS1 binding sites. As shown in **Table 1,** ORE1 binding specificity is highly similar to that or ORS1, and ORE1 tolerates a single-nucleotide mutation within the first (ORS1S11m2) or second binding motif (ORS1S11m1) without a significant reduction of its binding activity. Based on the ORS1 DNA-binding sequence profile and the binding site mutagenesis data presented in **Table 1**, the ORE1 binding site consists of two core motifs (motif 1, VMGTR; V = A, C or G; M = C or A; R = A or G) and (motif 2, YACR; Y = C or T), separated by a 5 - 6 bp long spacer.

In vitro Binding of ORE1 Transcription Factor to the *BFN1* Promoter

Regulation of gene expression involves the direct interaction of transcription factors with *cis*-regulatory elements located in the promoters of target genes. To investigate the physical interaction of ORE1 with the *BFN1* promoter we performed electrophoretic mobility shift assays (EMSAs). Sequence analysis of the *BFN1* promoter (1 kb upstream of the translation initiation site) revealed the presence of one full-length ORE1 binding site (called BS-1 in the following) -197 to -210 bp upstream the translation start site (**Figure 4A, Supplemental Table 4**); BS-1 differs at only a single position, within the second motif, from the consensus sequence. We tested interaction of recombinant ORE1 protein (fused to a glutathione S-transferase tag, ORE1-GST) with a 40-bp long double-stranded oligonucleotide harbouring BS-1 (5´-ACGTA(5n)CTCG-3´). ORE1-GST fusion protein (purified from recombinant *Escherichia coli*) was incubated with 5´-DY682-labeled 40-bp DNA fragment and analysed by EMSA. As shown in **Figure 4B** the DNA-protein complex migrated more slowly than free DNA indicating direct interaction of ORE1 with the labelled DNA. When unlabelled competitor DNA (oligonucleotide containing BS-1) was added in excess, a strong reduction in signal intensity was observed (**Figure 4B**), confirming specificity of the interaction.

Transactivation of the *BFN1* Promoter in Mesophyll Cell Protoplasts

To test whether *BFN1* is transactivated by ORE1, we performed luciferase-based transactivation assays using *Arabidopsis* mesophyll cell protoplasts. A reporter construct containing firefly luciferase (FLuc) coding region under the control of the ~1-kb *BFN1* promoter (*Prom$_{BFN1}$-FLuc*) (**Figure 4C**) was transformed into protoplasts in the presence or absence of *35S:ORE1* (**Figure 4C**); a significantly higher luciferase activity was observed when *Prom$_{BFN1}$-FLuc* was co-transformed with *35S:ORE1* than in controls that were solely transformed with the *Prom$_{BFN1}$-FLuc* construct, indicating that ORE1 transactivates *BFN1* expression in mesophyll cell protoplasts (**Figure 4D**). In the absence of *35S:ORE1*, only low basal luciferase activity was observed (**Figure 4D**). Next, we tested whether the ORE1 binding site (BS-1) present in the *BFN1* promoter is important for transactivation by the NAC factor. We performed two experiments: (i) we shortened the *BFN1* promoter down to ~200 bp and fused it to *FLuc*; this promoter lacked all upstream sequences including BS-1 (*Prom$_{BFN1}$-S-FLuc*) (**Figure 4C**). (ii) We mutated motif 1 of BS-1 within the 1-kb *BFN1* promoter (´CGT´ changed to ´AAA´; *Prom$_{BFN1}$-M-FLuc*; **Figure 4C**). As transactivation capacity of ORE1 was significantly reduced when BS-1 was mutated within the frame of the *BFN1* promoter (**Figure 4E**), we concluded that it is central for transactivation by ORE1. However, some transactivation was still observed with the *Prom$_{BFN1}$-M-FLuc* construct (**Figure 4E**). Analysis of the *BFN1* promoter revealed the presence of several partial ORE1 binding sites, all of which contained motif 1, but lacked motif 2 (**Supplemental Table 4**). The fact that some transcriptional activation of the *BFN1* promoter was still observed for the *Prom$_{BFN1}$-M-FLuc* construct may be due to the presence of these partial binding sites. Indeed, deletion of the *BFN1* promoter to ~200 bp which removed all sites strongly reduced *BFN1* transactivation (**Figure 4E**).

In Vivo Binding of ORE1 to the *BFN1* Promoter

To determine *in vivo* association of ORE1 with the *BFN1* promoter, we conducted chromatin immunoprecipitation together with quantitative PCR (ChIP-qPCR), using transgenic *Arabidopsis* lines expressing ORE1-GFP fusion protein from the CaMV 35S promoter. ORE1-GFP protein accumulated in nuclei of transgenic plants (**Figure 5A**) and showed elevated *BFN1* expression compared to wild type (**Figure 5B**), indicating that the ORE1-GFP fusion protein faithfully controlled its target gene. Using these plants we observed a significant enrichment of the *BFN1* promoter (**Figure 5C**), supporting the conclusion that it is a direct downstream target of ORE1. We also tested binding of ORE1 to the promoters of two

additional senescence-associated genes, i.e., *SAG29*/*SWEET15* and *SINA1* (**Supplemental Table 3**), and observed *in vivo* binding of the NAC transcription factor to both promoters (**Supplemental Figure 1**). In addition, ORE1 transactivates *SAG29* expression in mesophyll cell protoplasts (data not shown). *SAG29*/*SWEET15*, which encodes a non-characterized sugar transporter of the SWEET family (Chen et al., 2012), has recently been shown to accelerate senescence upon overexpression (Seo et al., 2011). Whether *SINA1*, which encodes a RING E3 ligase, also controls senescence is currently unknown.

Altered Level of BFN1 Protein in *ORE1* Transgenic Plants

Experiments were performed to examine whether altered expression of the *ORE1* transcription factor gene resulted in reduced BFN1 protein level, due to lowered *BFN1* expression. BFN1 levels were determined by Western blot in three different transgenic *Arabidopsis* lines in which *ORE1* expression was altered: the *anac092-1* knock-out mutant, the estradiol-inducible *ORE1-IOE* line, and the *35S:ORE1* overexpressor. Same-position leaves (6^{th} and 7^{th}) of the *anac092-1* knock-out mutant and wild-type Col-0 were detached and incubated in the dark until full senescence was obtained after 7 days in the wild type and 9 days in the mutant. Extracted proteins were used in Western blot experiments for measuring BFN1 levels. The results shown in **Figure 6A** demonstrate lack of BFN1 protein in the mutant compared to significant senescence-induced accumulation in the wild type. The consequence of short-term over-expression of *ORE1* on BFN1 protein levels was examined in the *ORE1-IOE* transgenic line. Seedlings were treated with estradiol for 24 h and the extracted proteins were analysed by Western blot. Clear induction of the BFN1 protein level was observed compared to wild-type *Arabidopsis* treated with estradiol (**Figure 6B**). In *35S:ORE1* plants constitutively over-expressing the transcription factor, full senescence was obtained within 6 days of incubation of detached leaves in the dark, while wild-type leaves incubated in parallel required 8 days to reach full senescence, indicated by complete yellowing of the leaf tissue. When BFN1 protein levels were measured in the senescing leaves a strongly elevated level was observed in the *35S:ORE1* overexpression lines (**Figure 6C**).

Discussion

The induction of senescence and its progression are highly regulated processes that involve the action of TFs as regulators of the expression of downstream target genes. Although many TFs are induced during senescence (e.g. Buchanan-Wollaston et al., 2005; Balazadeh et al.,

2008; Breeze et al., 2011), as shown by global transcriptome profiling in different plant species, only relatively few have been shown so far to indeed regulate senescence. Even less is known about the target genes or even entire gene regulatory networks through which these TFs exert their biological functions. One of the master transcriptional regulators of senescence in *Arabidopsis* is the NAC transcription factor ORE1 (ANAC092) (Kim et al., 2009; Balazadeh et al., 2010). *ORE1* expression increases in senescent leaves and mutants deficient for *ORE1* show delayed senescence (Kim et al., 2009; Balazadeh et al., 2010). Previously, we searched for genes affected by ORE1 by analyzing the expression profile of estradiol (EST)-inducible *ORE1* overexpression plants, 5 h after EST treatment. Expression of 170 genes was induced by ORE1, of which 102 were previously reported as senescence-associated genes (SAGs; Balazadeh et al., 2010). Biocomputational analysis and network modelling based on high-resolution time-course profiles of gene expression during development predicts a positive influence of ORE1 on several of the genes that showed increased expression after EST-treatment in *ORE1-IOE* plants (Balazadeh et al., 2010; Breeze et al., 2011). However, direct or indirect regulation can only be distinguished by additional laboratory experiments.

BIFUNCTIONAL NUCLEASE1 (BFN1) has been associated with nucleic acid degradation during senescence (Pérez-Amador et al., 2000; Farage-Barhom et al., 2008; Farage-Barhom et al., 2011), however, its integration into a gene regulatory network has not been known so far. In this study we identified *BFN1* as a direct target of ORE1. *BFN1* transcript level rapidly increases upon induction of *ORE1*, both in intact *Arabidopsis* plants and in isolated mesophyll cell protoplasts. In addition, ORE1 binds *in vivo* to the *BFN1* promoter, as shown be chromatin immunoprecipitation (**Figure 5C**). Accordingly, *BFN1* expression in senescing leaves and flower abscission zones is completely lost in mutants lacking functional *ORE1*, as shown by qRT-PCR and *BFN1* promoter - GUS reporter studies. The strong decrease of *BFN1* expression in the *ore1-2* and *anac092-1* mutants is consistent with the model that ORE1 is the major, if not the only upstream TF that regulates it, at least in the tissues analysed here (**Figure 7**). Furthermore, in aging leaves *BFN1* was expressed earlier and at higher levels in the *mir164abc* mutant than in the wild type, in accordance with the observation that *miR164* negatively regulates *ORE1* transcript abundance (Kim et al., 2009). Thus, *BFN1* expression is under indirect control of *miR164*, which mediates its effect on *BFN1* through regulation of *ORE1* transcript abundance.

We show here that ORE1 best binds to a bipartite *cis*-element that contains two core motifs (motif 1, VMGTR) and (motif 2, YACR) separated by 5 or 6 bp (**Table 1**). Reducing or increasing the distance between the two motifs to 4 or 7 bp, respectively, strongly reduces binding affinity of ORE1 to double-stranded DNA *in vitro*, similar to ORS1 (**Table 1**; see also Balazadeh et al., 2011), the closest homolog of ORE1 in *Arabidopsis* (Ooka et al., 2003). Notably, the two motifs establish a semi-palindromic ORE1 binding site. Earlier, Olsen et al. (2005), using *in vitro* binding site selection, identified the 9-mer sequence T[TAG][GA]CGT[GA][TCA][TAG] with only one motif (underlined) as the ORE1 binding site. Although a second motif was not discovered in these experiments (probably due to the design of the oligonucleotides used; Olsen et al., 2005), experimental evidence shows that binding affinity of ORE1 and ORS1 transcription factors to DNA benefits from the presence of motif 2, if separated by a fixed number of base-pairs (**Table 1**; Balazadeh et al., 2010). In accordance with this, Jensen et al. (2010) have recently demonstrated binding of ORE1, ANAC019 (At1g52890), ATAF1 (ANAC002; At1g01720) and VND7 (ANAC030; At1g71930) and some other NACs to palindromic NAC transcription factor binding sites. Bipartite binding sites were also reported for other NACs including TaNAC69 from wheat (Xue et al., 2005), the secondary well-associated *Arabidopsis* NACs SND1, VND6, VND7, NST1 and NST2 (Zhong et al., 2010), and the *Arabidopsis* negative senescence regulator JUNGBRUNNEN1 (JUB1; Wu et al., 2012).

A recent report provided evidence that *BFN1* might also be regulated by SND1 (ANAC012; At1g32770) and VND7 (ANAC030; At1g71930) (Zhong et al., 2010). However, as neither of the two genes is upregulated during leaf senescence (and as both are only weakly expressed in non-senescent leaves), it appears unlikely that they serve as upstream activators of *BFN1* in this process. Still, it may be possible that the two NAC factors control *BFN1* expression during secondary wall formation in which both, SND1 and VND7 play important roles (Kubo et al., 2005; Zhong et al., 2006). Indeed, BFN1 harbours three potential SND1-binding sites in its 1-kb 5´ upstream promoter, in addition to the ORE1 binding sites (**Supplemental Table 4**).

Currently, knowledge about genes directly or indirectly regulated by senescence-associated NAC transcription factors is increasing. We previously identified genes early responding to *ORS1* induction. Although *ORS1* is the paralog of *ORE1* (Ooka et al., 2003), only few genes (eight in total) were affected by both TFs, indicating minimal overlap between the downstream regulatory pathways administrated by ORE1 and ORS1. Of note, *BFN1* was not

among the genes affected by ORS1 (Balazadeh et al., 2011), indicating its specific regulatory link to ORE1. The VNI2 transcription factor has been shown to directly regulate expression of *COR* (*COLD-REGULATED*) and *RD* (*RESPONSIVE TO DEHYDRATION*) genes and to be involved in the crosstalk between stress signals and aging programs (Yang et al., 2011). Recently, *DREB2A* and *SAG113* were reported as direct targets of JUB1 and AtNAP, respectively (Wu et al., 2012; Zhang et al., 2012). None of these genes has been shown to be regulated by other so far characterized senescence-associated NAC factors.

Although in this report we focused our analysis on *BFN1* as a target gene of ORE1, we have shown that it robustly activates the expression of several other downstream genes (**Supplemental Table 3**). In addition, we have demonstrated that the promoters of two further senescence-associated genes, i.e., *SAG29*/*SWEET15* and *SINA1*, are bound by ORE1 *in vivo*. In addition, *SAG29* promoter activity is activated by ORE1 in a mesophyll cell protoplast transactivation assay (not shown), similar to *BFN1* (**Figure 4D,E**). Plants lacking functional *BFN1* do not appear to exhibit a remarkable change in senescence, which may be due to the fact that the senescence syndrome results from a complex interplay of many metabolic and structural changes, and altering individual elements may not always have a strong enough impact on the entire process, which must be buffered against over-rapid tissue deterioration to allow plants a coordinated recovery of leaf carbon and nitrogen to feed newly developing organs (e.g. young leaves, flowers) during senescence. Still, it has been clearly demonstrated that during the late stage of senescence the BFN1 nuclease localizes together with fragmented nuclei in membrane-coated vesicles, which indicates a role for BFN1 in regulated nucleic acid breakdown while senescence progresses (Farage-Barhom et al., 2011). In addition, *SAG29*/*SWEET15* has recently been demonstrated to accelerate senescence when overexpressed (Seo et al., 2011), possibly by altering sugar transport (as *SWEET15* encodes a sugar transporter family protein).

In conclusion, our results reveal NAC transcription factor ORE1 as a central, and most likely sole, upstream transcriptional regulator of the nuclease-encoding gene *BFN1* during leaf senescence in *Arabidopsis thaliana*, suggesting that part of the ORE1-controlled senescence syndrome involves nucleic acid degradation. Furthermore, ORE1 directly regulates the expression of two additional senescence-associated genes (*SAG29* and *SINA1*), supporting the important role of ORE1 in senescence control. Although a direct upstream regulator of *ORE1* has not been reported yet, experimental evidence points to an involvement of ethylene

signalling (He et al., 2005; Kim et al., 2009). In addition, *miR164* controls *ORE1* transcript abundance (Kim et al., 2009) and thus indirectly impinges on *BFN1* expression.

METHODS

General

Standard molecular techniques were performed as described (Sambrook et al., 2001; Skirycz et al., 2006). All chemicals and reagents were obtained from Sigma-Aldrich (Deisenhofen, Germany), Fluka (Buchs, Switzerland) or Merck (Darmstadt, Germany). Molecular biological reagents were purchased from Invitrogen (Karlsruhe, Germany), Applied Biosystems (Darmstadt, Germany), Stratagene (Heidelberg, Germany), or Roche (Mannheim, Germany). Molecular kits were obtained from Qiagen (Hilden, Germany) and Macherey-Nagel (Düren, Germany). Oligonucleotides were synthesized by MWG (Ebersberg, Germany) or GeneWorks (Adelaide, Australia). DNA sequencing was performed by MWG and GeneWorks. For sequence analysis the tools provided by the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), MIPS (http://mips.gsf.de/), European Bioinformatics Institute (http://www.ebi.ac.uk/) and the Arabidopsis Information Resource (TAIR; http://www.arabidopsis.org/) were used.

Plant Material and Growth Conditions

Seeds of *Arabidopsis thaliana* (L.) Heynh. ecotype Col-0 were obtained from the *Arabidopsis thaliana* Resource Centre for Genomics (INRA, France; http://dbsgap.versailles.inra.fr/publiclines/). The *anac092-1* T-DNA insertion line was originally obtained from the SALK collection (ID 090154) and was reported earlier (Balazadeh et al., 2010). For the experiment shown in **Figure 3** we used RNA from the *ore1-2* and *miR164abc* mutants reported by Kim et al. (2009). Generation of the $Prom_{BFN1}$*:GUS* transgenic lines was reported previously (Farage-Barhom et al., 2008). Seeds were surface-sterilized for 1 min in 70% ethanol and then in sterilisation solution (20% sodium hypochlorite) for 30 min. After sterilization, the seeds were sown on half-strength MS medium (Murashige and Skoog, 1962), supplemented with 1% (w/v) sucrose and solidified with 0.7% (w/v) phytoagar and appropriate antibiotics. After imbibition, the seeds were stratified at 4°C for 3 days. The seeds were germinated at 22°C under a 16-h day (140 µmol m^{-2} s^{-1}) / 8-h night regime. The two-week-old *Arabidopsis* seedlings were carefully removed

from plates and transplanted to soil (Einheitserde GS90; Gebrüder Patzer, Sinntal-Jossa, Germany) or, if necessary, directly subjected to various treatments. Unless otherwise indicated, *Arabidopsis* plants were grown in controlled conditions in a growth chamber with 16-h day length provided by fluorescent light at 80 or 120 µmol m^{-2} s^{-1}, a day/night temperature of 20/16°C and relative humidity of 60/75%. *Agrobacterium tumefaciens* strain GV3101 (PMP90) was used for *Arabidopsis thaliana* transformations.

Dark-Induced Senescence

Experiments for artificial induction of senescence were performed with leaves in position 6 or 7 of the *Arabidopsis* plants. The leaves were detached and incubated in the dark in containers fitted with inlet and outlet ports, which were stored for 6 - 9 days in the dark at 25°C. The containers were sealed and connected to a flow-through air supply, which was bubbled through sterile water to maintain humidity.

Constructs

Primer sequences are given in **Supplemental Table 5.** *ORE1-CELD*: *ORE1* cDNA PCR-amplified from *Arabidopsis* leaf cDNA with primers *ORE1-CELD*-fwd and *ORE1-CELD*-rev was inserted into pCR2.1–TOPO and then cloned via *Nhe*I and *Bam*HI sites into plasmid pTacLCELD6XHis (Xue, 2005) to create an ORE1-CELD in-frame fusion construct (pTacORE1LCELD6XHis). *$Prom_{BFN1}$-FLuc:* The ~1.0-kb *BFN1* promoter and a ~0.2 kb long truncated version (counted from the translation initiation codon) were amplified by PCR from *Arabidopsis* genomic DNA and inserted into pENTR/D-TOPO vector (Invitrogen). The mutated *BFN1* promoter which contained a CGT-to-AAA sequence change within the ORE1 BS-1 was generated by overlap PCR (for primer sequences see **Supplemental Table 5**). The sequence-verified entry clones were then transferred to the p2GWL7.0 vector (Ghent University; http://gateway.psb.ugent.be/vector) harbouring the firefly luciferase (FLuc) coding region by LR recombination to generate *$Prom_{BFN1}$-FLuc* (full length *BFN1* promoter), *$Prom_{BFN1}$-M-FLuc* (full length *BFN1* promoter with mutated ORE1 binding site) and *$Prom_{BFN1}$-S-FLuc* (short promoter). *35S*:*ORE1-GFP*: the full-length *ORE1* open reading frame was amplified without its stop codon. The PCR product was cloned into the pENTR/D-TOPO vector using the pENTR Directional TOPO cloning kit (Invitrogen). The sequence-verified entry clone was then transferred to the pK7FWG2 vector (Karimi *et al*., 2002) by LR recombination (Invitrogen). The *35S:ORE1* and *ORE1-IOE* lines used here are identical to the

35S:ANAC092 and *ANAC092-IOE* lines, respectively, reported earlier (Balazadeh et al., 2010).

Transformation of the *anac092-1* Mutant With the *Prom$_{BFN1}$:GUS* Construct

The *Prom$_{BFN1}$:GUS* construct (Farage-Barhom et al., 2008) was introduced into the *anac092-1* T-DNA insertion mutant by *Agrobacterium*-mediated transformation and T0 seedlings were selected on kanamycin (50 mg L^{-1}). Kanamycin-resistant lines were analysed by PCR for GUS reporter gene-specific amplification. Tissue-specific expression was analysed using plants of the T3 generation.

Transient Expression of *ORE1* in Protoplasts for Transcriptome Profiling

The protoplast preparation protocol was adapted from Sheen (2002). *Arabidopsis* mesophyll cell protoplasts were isolated from leaves (the second and/or third/fourth pair) of 5-week-old Col-0 plants grown in soil under long-day (16 h light / 8 h dark) condition. Leaves were placed in enzyme solution (1 % cellulase R10, 0.3% macerozyme R10 [Yakult Honsha, Tokyo, Japan], 0.4 M mannitol, 20 mM KCl, 10 mM $CaCl_2$, 20 mM MES, 0.1% BSA [Sigma A-6793), pH 5.7]) for 8.5 h. Protoplasts were collected and kept on ice in W5 medium (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7) for 13 h in the growth cabinet. Protoplasts were transferred to MMG solution (0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7), and subjected to PEG transfection. To 5 x 10^6 protoplasts, a total of 250 µg plasmid DNA was added followed by 30 min incubation in 1 vol. of PEG solution (40% PEG 3500, 3 mL H_2O, 0.2 M mannitol, 0.1 M $CaCl_2$). After transfection the samples were diluted with 2 vol. of W5 solution and collected by centrifugation at 100 *g* for 2 min. Protoplasts were then resuspended in 4 mL WI medium (0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7), transferred to 5-cm Petri dishes pre-coated with 5% calf serum, and incubated for 6 h in the growth cabinet. After the incubation, protoplasts were collected and 100 - 200 mg aliquots were flash-frozen in liquid nitrogen for subsequent RNA isolation and expression profiling.

ORE1 Binding Assay

The DNA-binding activity of ORE1-CELD fusion protein was measured using methylumbelliferyl β-D-cellobioside (MUC) as substrate (Xue, 2002). DNA-binding assays

with a biotin-labelled single-stranded oligonucleotide or a biotin-labelled double-stranded oligonucleotide without a target binding site were used as controls.

Gene Expression Analysis by Microarray Hybridisation

Three micrograms of quality-checked total RNA obtained from 3-week-old *ORE1-IOE* seedlings (2 h after 10 µM estradiol treatment or 0.15% ethanol for control) and of mesophyll cell protoplasts transfected with the *35S:ORE1* construct were used for Affymetrix ATH1 micro-array hybridisations (two biological replicates each). Labelling, hybridisation, washing, staining, and scanning procedures were performed by ATLAS Biolabs (Berlin, Germany)**.** Data analysis was performed as described (Balazadeh et al., 2010). Expression data (*ORE1-IOE-2h* and *35S:ORE1-6h* datasets) were submitted to the NCBI Gene Expression Omnibus (GEO) repository (www.ncbi.nlm.nih.gov/geo/) under accession number GSE37536.

Gene Expression Analysis by Quantitative RT-PCR

Quantitative RT-PCR was done as described (Caldana et al., 2007; Balazadeh et al., 2008). Primers were designed using QuantPrime (Arvidsson et al., 2008). PCR reactions were run on ABI PRISM 7900HT sequence detection system (Applied Biosystems), and amplification products were visualized using SYBR Green (Applied Biosystems). Data were normalized to reference genes *ACTIN2* (At3g18780).

Histochemical and Quantitative GUS Assays

Histochemical GUS assays were performed as described (Plesch et al., 2001). Fluorometric GUS assays were performed using 4-methylumbelliferyl β-D-glucuronide (4-MUG; Sigma-Aldrich) as substrate (Jefferson et al., 1987).

Protein Expression and Electrophoretic Mobility Shift Assay (EMSA)

For protein expression and purification, the *ORE1* cDNA was recombined into the Gateway vector pDEST24 (Invitrogen) encoding a C-terminal GST-tag, and transformed into *E. coli* strain BL21 (DE3) pLysS (Agilent Technologies, Waldbronn, Germany). pDEST15 vector (Invitrogen) was used for expression of GST alone. Protein expression in 400-mL cultures was induced at 30°C by 1 mM isopropyl thio-β-D-galactoside for 3 h. Cells were harvested and lysed by ultrasound in 20 mL GST lysis buffer (20 mM sodium phosphate buffer, pH 7.3, 150 mM NaCl, 1 mM EDTA, 0.2% Triton X-100, 1 mM dithiothreitol, 1 mM

phenylmethanesulfonyl fluoride, 10 µg mL^{-1} aprotinin, 10 µg mL^{-1} leupeptin, 2 mM benzamidin). The supernatant of ultra-centrifuged samples was used for affinity purification with 70 mg of pre-equilibrated glutathione-agarose beads (Sigma-Aldrich). Aliquots of the elution fractions were analysed by SDS-PAGE and Coomassie staining. One-mL fractions containing the purified proteins were pooled and dialyzed against PBS buffer (20 mM sodium phosphate buffer, pH 7.4, 150 mM NaCl). Concentrations of purified proteins were determined by SDS-PAGE and Coomassie staining using BSA standard. EMSAs using 5´-DY682-labelled DNA fragments were performed as reported (Wu et al., 2012).

Transactivation Assay

Assays were essentially performed as described (Wu et al., 2012), using *35S:ORE1* plasmid (Balazadeh et al., 2010) as effector. Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega) and a GloMax 20/20 Luminometer (Promega). All tests were performed in 3 - 4 independent biological replications with three technical replications per assay.

ChIP-qPCR

Shoots from 15-day-old *Arabidopsis* plants expressing GFP-tagged ORE1 protein from the CaMV 35S promoter (*35S*:*ORE1-GFP*) were used for ChIP-PCR. *35S:JUB1-GFP* plants (Wu et al., 2012) were used as control. ChIP was done as reported (Lai et al., 2012). Anti-GFP antibody (Roche Applied Science, Germany) was used for immunopurification of ORE1-GFP-DNA or JUB1-GFP-DNA complexes. qPCR was performed using primers flanking the ORE1 binding site (BS-1) of the *BFN1* promoter, or ORE1 binding sites of the *SAG29* or *SINA1* promoters. Primers detecting enrichment of a promoter lacking both, ORE1 and JUB1 binding sites (At2g22180) were used for negative control. The amount of genomic DNA co-precipitated by GFP antibody (ChIP signal) was calculated in comparison to the total input DNA used for each immunoprecipitation in the following way: cycle threshold (C_T) = $C_{T(ChIP)}$ − $C_{T(Input)}$. To calculate fold enrichment, normalized ChIP signals were compared between *35S:ORE1-GFP* and *35S:JUB1-GFP* (Wu et al., 2012), where the ChIP signal is given as the fold increase in signal relative to the background signal.

Western Blot Analysis

BFN1 polyclonal antibodies were raised against an over-expressed portion of the protein using the bacterial pET system (Novagen, Madison, USA) basically as described before (Lers et al., 2006). Part of the *BFN1* coding region (nucleotide position 162 to 876) was PCR amplified using the primers BFNexprev (5'-GGATCCCGGTTTAGTATCATGGCTAGT-3') and BFNexpforw (5'-GGATCCGTTACCGGATTACGTGAAAGG-3'). The *BFN1* fragment was fused via *Bam*HI to the His tag-coding segment in the pET-22b(+) vector (Novagen); the BFN1-His protein was expressed in *Escherichia coli*, purified on His bind Quick 900 cartridge (Novagen), and used for immunization of rabbits.

Western blot analysis was essentially performed as described after separation of proteins on 15% SDS-polyacrylamide gels (Lers et al., 2006). Goat anti-rabbit IgG : horseradish peroxidase conjugate (Bio-Rad) was used as secondary antibody. For signal detection, WesternBright ECL kit (Advansta, CA, USA) was used.

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AUTHOR CONTRIBUTIONS

BMR and SB designed the research and supervised the group. LMR and SB performed the research. MR tested expression of *ORE1* and *SAG12* in *ore1-2* and *mir164abc* mutants. GPX performed the CELD experiment. WDL performed transient overexpression of *ORE1* in *Arabidopsis* protoplasts. AL provided seeds of the $Prom_{BFN1}$*:GUS* lines; AL and SFB carried out immunoblotting experiments. HD produced recombinant ORE1-GST protein. SB and BMR wrote the article with contributions from the other authors.

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Table 1. Binding of ORE1 to Perfect and Mutated ORS1 Binding Sites. Relative binding activity (RBA) of ORE1 to oligonucleotide ORS1S11 is set to 1.

Synthetic oligonucleotide	Sequence	RBA (ORS1)	RBA (ORE1)
ORS1S11	CGGGGTTACGTACGGCACACGCAACCGTGC	1.00 ± 0.07	1.00 ± 0.09
ORS1S11m1	CGGGGTTACGTACGGCACACaCAACCGTGC	0.29 ± 0.02	0.63 ± 0.01
ORS1S11m2	CGGGGTTAaGTACGGCACACGCAACCGTGC	0.52 ± 0.05	0.93 ± 0.11
ORS1S11m3	CGGGGTTgCGTACGGCACACGCAACCGTGC	1.13 ± 0.02	1.12 ± 0.18
ORS1S11m4	CGGGGTTACGTACGGCACACGtAACCGTGC	1.08 ± 0.08	1.20 ± 0.20
ORS1S11m5	CGGGGTTACGTA()GGCACACGCAACCGTGC	0.04 ± 0.01	0.17 ± 0.01
ORS1S11m6	CGGGGTTACGTA(C)CGGCACACGCAACCGTGC	1.09 ± 0.02	1.07 ± 0.06
ORS1S11m7	CGGGGTTACGTA(CT)CGGCACACGCAACCGTGC	0.11 ± 0.02	0.33 ± 0.01
F17d127	TGCCCAATGCCGTGTGTAGCACGCTGCCCA	n.d.	0.63 ± 0.01

ORS1S11 represents the ORS1-selected oligonucleotide, while all other oligonucleotides, with the exception of F17d127, represent mutations thereof. F17d127 was included in the assay as it was previously found to be targeted by NAC transcription factor JUB1 (Wu et al., 2012). The two binding motifs are underlined. Point mutations in ORS1S11-derived oligonucleotides are indicated by lower-case letters, deletions by empty brackets, and insertions by filled brackets with the added nucleotides indicated. Values are means ± SD of three assays. n.d., not determined.

Figures.

Figure 1.

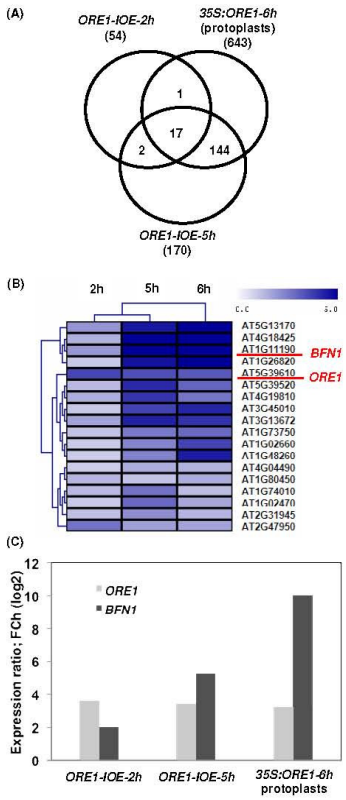

Figure 1. Overview of ORE1 Early Induced Genes.

(A) Venn diagrams of genes upregulated 2 h and 5 h, respectively, after EST treatment in *ORE1-IOE* plants (*ORE1-IOE-2h* and *ORE1-IOE-5h* datasets) and 6 h after the transfection of mesophyll cell protoplasts with the *35S:ORE1* construct (*35S:ORE1-6h* dataset), as identified by Affymetrix ATH1 hybridisation experiments. The numbers of genes altered relative to control in the three datasets are given in brackets. **(B)** Hierarchical clustering of 17 putative direct target genes of ORE1. Clusters were generated from expression data obtained from the *ORE1-IOE-2h, ORE1-IOE-5h* and *35S:ORE1-6h* datasets. Gene expression ratios: dark blue, maximum (5.0: 32-fold upregulation). AGI codes of *BFN1* and *ORE1/ANAC092* are underlined. **(C)** *BFN1* expression is elevated upon induction of *ORE1*. Data were extracted from the ATH1 hybridisations (*ORE1-IOE-2h*, *ORE1-IOE-5h* and *35S:ORE1-6h* datasets) and represent the means of two biological replicates each. FCh, fold change.

Figure 2.

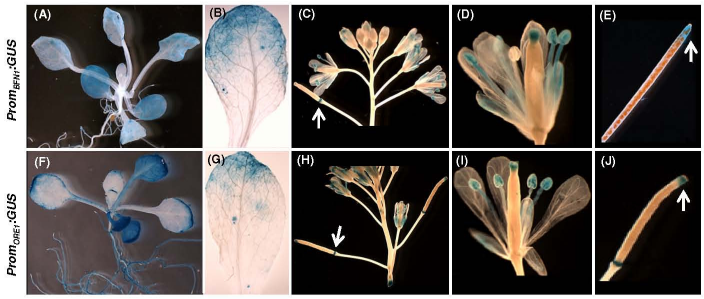

Figure 2. Overlap of *ORE1* and *BFN1* Expression Patterns.

Histochemical staining of transgenic plants carrying the *GUS* reporter gene downstream of the *BFN1* (**A-E**) or *ORE1* (**F-J**) promoters revealed a prominent overlap of GUS staining in vegetative and floral organs. (**A-F**) Fifteen-day-old seedlings. Strong GUS staining is present in cotyledons and tips and margins of older leaves. (**B** and **G**) GUS staining in senescent regions of 20 - 30% yellow leaves from 40-day-old plants. **(C, D, H** and **I)** Floral organs. GUS staining was only observed in mature open flowers (stage 13 - 14), but not in immature flowers. (**D and I**) Strong GUS staining in stigma and mature anthers, and weaker staining in sepals. (**C, H, E** and **J**) GUS activity in abscission zones and the tip regions of developed siliques (arrows). The silique shown in **(J)** is a magnification of a silique shown in **(H)**.

Figure 3.

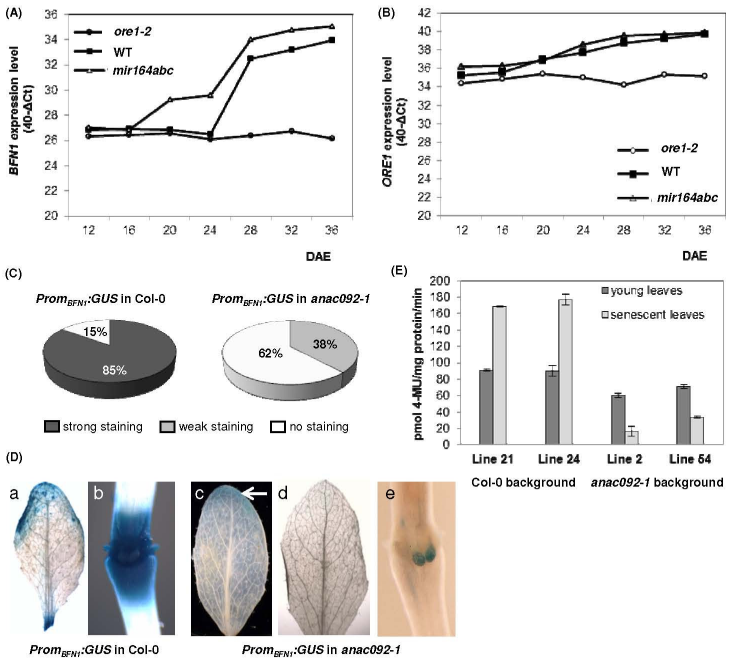

Figure 3. *BFN1* Expression in Wild-type Plants and *ore1* Mutants.

(**A**) *BFN1* transcript levels in leaves of *ore1-2*, *mir164abc* and wild-type (WT) *Arabidopsis* plants (Kim et al., 2009) at different ages (12, 16, 20, 24, 28, 32 and 36 days after emergence, DAE). (**B**) *ORE1* expression in the same samples as in (A). Note the absence of *BFN1* expression change in leaves of the *ore1-2* mutant, and an earlier increase of *BFN1* expression in the *mir164abc* mutant. Expression levels in (A) and (B) were determined by qRT-PCR and data are means of two biological replicates each. (**C**) to (**E**) $Prom_{BFN1}$*:GUS* reporter activity in senescent leaves and the silique abscission zone. The $Prom_{BFN1}$*:GUS* reporter construct was transformed into wild-type (Col-0) and *anac092-1* mutant plants and GUS activity patterns were analysed. **(C)** Percentage of $Prom_{BFN1}$*:GUS*-transformed Col-0 and *anac092-1* mutant lines showing high, weak or no GUS activity in senescent leaves (~20% yellowing, visually inspected) of 40-day-old plants. Thirty and 50 independent $Prom_{BFN1}$*:GUS* transformants in Col-0 or *anac092-1* background were tested, respectively. GUS staining intensity was scored visually. **(D)** Strong GUS staining in the tip region of a senescent leaf (a) and the flower abscission zone (b) of a $Prom_{BFN1}$*:GUS* transformed Col-0 plant. From (c) to (e): Residual

GUS staining in the *anac092-1* mutant transformed with the *Prom$_{BFN1}$:GUS* construct. Leaves with weak (c; line 54; arrow) or no detectable *BFN1* expression (d; line 2) are shown. Panel (e) shows residual GUS activity in the flower abscission zone. The images show representative examples of at least 30 independent transgenic lines examined per transformation. **(E)** GUS activity of *Prom$_{BFN1}$:GUS*-transformed lines measured by MUG assay in leaves of young and old leaves of 20- and 40-day-old transgenic lines. Lines 21 and 24 are in Col-0, lines 2 and 54 in *anac092-1* background. Data are the means ± SD of two independently performed biological experiments, which included three measurements for each experiment.

Figure 4.

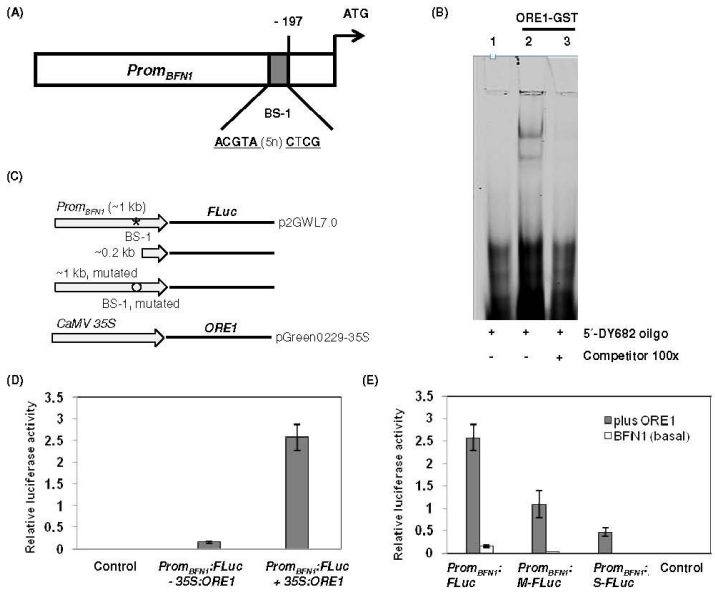

Figure 4. EMSA and Transactivation Assay.

(A) Schematic diagram of the *BFN1* promoter (~1 kb upstream the translation start site, ATG) showing the ORE1 binding site 1 (BS-1). **(B)** Electrophoretic mobility shift assay. Purified

ORE1-GST protein binds *in vitro* to the 40-bp sequence of the *BFN1* promoter, harbouring BS-1 (band shift seen in lane 2). No band shift is observed in the absence of ORE1-GST protein (lane 1) or when unlabelled fragment (100-fold excess) is used to compete with 5´-DY682-labelled probe (lane 3). **(C)** Diagram of the constructs used for the transactivation assays. Positions of the wild-type and mutated ORE1 BS-1 binding sites are indicated. **(D)** and **(E)** Firefly luciferase reporter activities in *Arabidopsis* (Col-0) mesophyll cell protoplasts. **(D)** Luciferase activity driven by the ~1 kb $Prom_{BFN1}$ promoter (*$Prom_{BFN1}$-FLuc*) in the absence or presence of *35S:ORE1* effector plasmid. **(E)** Luciferase activity driven by the wild-type ~1 kb $Prom_{BFN1}$ promoter (*$Prom_{BFN1}$-FLuc*), the shortened, ~200 bp promoter (*$Prom_{BFN1}$-S-FLuc*), or the mutated ~1 kb $Prom_{BFN1}$ promoter lacking BS-1 (*$Prom_{BFN1}$-M-FLuc*) in the presence (grey columns) or absence (white columns) of *35S:ORE1* effector plasmid. Luciferase activity was determined using the Dual Luciferase Reporter Assay System (Promega) 24 h after transfection. To normalize for transformation efficiency, the CaMV *35S:RLuc* plasmid was co-transformed. ´Control´ indicates data from non-transformed protoplasts. Data represent means ± SD of three biological replicates.

Figure 5.

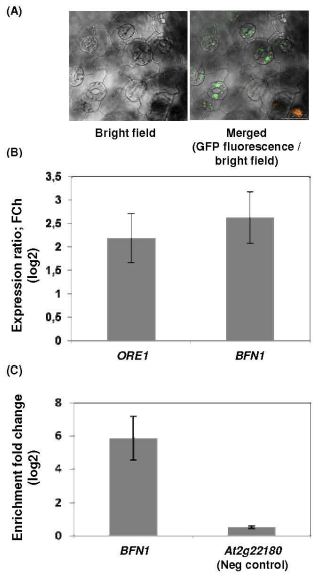

Figure 5. ***In vivo*** **Interaction of ORE1 With *BFN1* Promoter.**

(A) Nuclear localization of ORE1-GFP fusion protein in transgenic *Arabidopsis* plants expressing GFP-tagged ORE1. **(B)** Expression of *ORE1* and *BFN1* in 15-day-old *35S:ORE1-GFP* plants compared with wild type (WT); *ORE1* expression represents the combined expression of the endogenous *ORE1* gene and the *ORE1-GFP* transgene. Numbers on the Y axis indicate expression fold change (log2 basis) compared with WT. Data are the means ± SD of two biological replicates each determined in three technical replicates. **(C)** ChIP-qPCR. Shoots of 15-day-old *35S:ORE1-GFP* and *35S:JUB1-GFP* (control) seedlings were harvested for the ChIP experiment. qPCR was used to quantify enrichment of the *BFN1* promoter. As negative control, primers annealing to a promoter region of an *Arabidopsis* gene lacking both, ORE1 and JUB1 binding sites (*At2g22180*) were employed. To calculate fold enrichment (Y axis), normalized ChIP signals were compared between *35S:ORE1-GFP* and *35S:JUB1-GFP* (Wu et al., 2012). Data represent means ± SD of three independent experiments.

Figure 6.

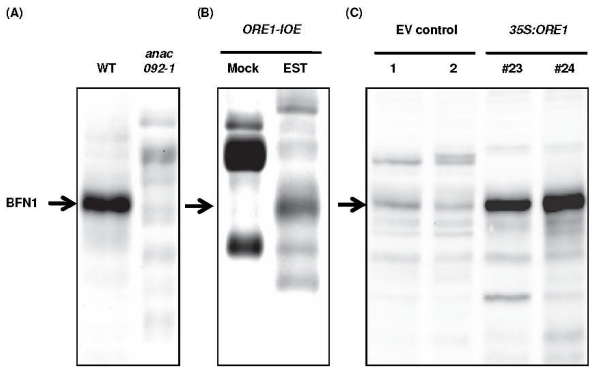

Figure 6. The effect of Modulating *ORE1* Expression on BFN1 Protein Levels.

(A) BFN1 protein level in the *anac092-1* knock-out mutant. Leaves of the *Arabidopsis* wild-type (WT) and *anac092-1* mutant line were subjected to artificial senescence. Proteins (10 µg) extracted from fully senescent leaves were used for Western blot analysis to determine BFN1 protein level. **(B)** BFN1 protein level following regulated induction of the *ORE1* gene. *ORE1-IOE* seedlings were subjected to 10 µM estradiol (EST) treatment for 24 h or were not treated (Mock). Extracted proteins (10 µg) were used for Western blot analysis to determine BFN1 protein level. While the BFN1 polyclonal antibodies cross-react non-specifically with proteins of young leaf tissue, activation of ORE1 which induces senescence results in a decrease of the non-specific background signal. **(C)** BFN1 protein level in plants constitutively over-expressing *ORE1*. Leaves of two different empty vector (EV)-transformed control lines (´1´ and ´2´) and of two independent *35S:ORE1* lines (lines 23 and 24) were subjected to artificial senescence. Proteins (10 µg) extracted from fully senescent leaves were used for Western blot analysis to determine BFN1 protein level. In all three panels the position of the BFN1 protein is indicated by arrows.

Figure 7.

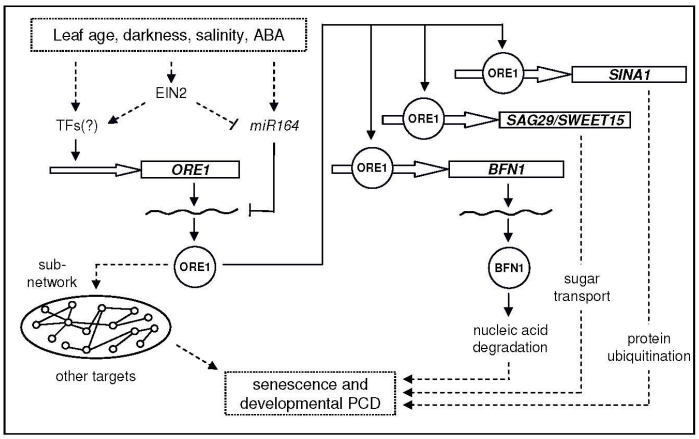

Figure 7. Schematic Model Highlighting the ORE1-*BFN1* Regulatory Network During Senescence.

Developmental and environmental signals trigger *ORE1* expression. *ORE1* expression is upregulated with leaf age which involves EIN2 (ETHYLENE INSENSITIVE2), but is negatively regulated by *miR164* (Kim et al., 2009). ORE1 controls developmental senescence and PCD through a gene regulatory network that includes *BFN1* and other direct target genes, including *SAG29*/*SWEET15* and *SINA1*. BFN1 plays a role in nucleic acid degradation that takes place during senescence and PCD.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

Supplemental Figure 1. *In Vivo* Binding of ORE1 to the Promoters of *SAG29* and *SINA1* Demonstrated by Chromatin Immunoprecipitation Analysis.

Supplemental Table 1. Genes differentially expressed upon transient overexpression of *ORE1* in *Arabidopsis* mesophyll cell protoplasts.

Supplemental Table 2. Genes affected in *Arabidopsis ORE1-IOE* plants 2 h after estradiol treatment.

Supplemental Table 3. Genes commonly upregulated upon elevated *ORE1* expression in stably transformed plants and transiently transfected mesophyll cell protoplasts.

Supplemental Table 4. ORE1 binding sites in *BFN1* promoter.

Supplemental Table 5. Oligonucleotide sequences.