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ORS1, an H_2O_2-Responsive NAC Transcription Factor, Controls Senescence in *Arabidopsis thaliana*

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ABSTRACT We report here that ORS1, a previously uncharacterized member of the NAC transcription factor family, controls leaf senescence in *Arabidopsis thaliana*. Overexpression of *ORS1* accelerates senescence in transgenic plants, whereas its inhibition delays it. Genes acting downstream of ORS1 were identified by global expression analysis using transgenic plants producing dexamethasone-inducible ORS1–GR fusion protein. Of the 42 up-regulated genes, 30 (~70%) were previously shown to be up-regulated during age-dependent senescence. We also observed that 32 (~76%) of the ORS1-dependent genes were induced by long-term (4 d), but not short-term (6 h) salinity stress (150 mM NaCl). Furthermore, expression of 16 and 24 genes, respectively, was induced after 1 and 5 h of treatment with hydrogen peroxide (H_2O_2), a reactive oxygen species known to accumulate during salinity stress. *ORS1* itself was found to be rapidly and strongly induced by H_2O_2 treatment in both leaves and roots. Using *in vitro* binding site selection, we determined the preferred binding motif of ORS1 and found it to be present in half of the ORS1-dependent genes. *ORS1* is a paralog of *ORE1*/*ANAC092*/*AtNAC2*, a previously reported regulator of leaf senescence. Phylogenetic footprinting revealed evolutionary conservation of the *ORS1* and *ORE1* promoter sequences in different Brassicaceae species, indicating strong positive selection acting on both genes. We conclude that ORS1, similarly to ORE1, triggers expression of senescence-associated genes through a regulatory network that may involve cross-talk with salt- and H_2O_2-dependent signaling pathways.

Key words: NAC transcription factor; leaf senescence; gene expression; gene regulatory network; hydrogen peroxide.

INTRODUCTION

Leaf senescence is controlled by organ age and is triggered by adverse environmental factors (e.g. Lutts et al., 1996; Pourtau et al., 2004; Munns, 2005; Masclaux-Daubresse et al., 2007). Additionally, plant growth regulators such as ethylene, salicylic acid, jasmonic acid, auxin, abscisic acid, and cytokinins affect senescence (Lim et al., 2007). Onset and progression of senescence are accompanied by global changes in gene expression (e.g. Gepstein et al., 2003; Andersson et al., 2004; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Lim et al., 2007; Balazadeh et al., 2008b). Genes up-regulated during senescence are generally termed senescence-associated genes (SAGs). Transcription factors (TFs) of the NAC (for NAM, ATAF1, 2, and CUC2) domain family represent an appreciable portion of the senescence-regulated genes in many plant species including crops and trees, suggesting an important role in the control of senescence (e.g. Andersson et al., 2004; Guo et al., 2004; Buchanan-Wollaston et al., 2005; Gregersen and Holm, 2007; Balazadeh et al., 2008b).

In *Arabidopsis thaliana*, expression of more than 20 NAC TFs increases during senescence (Buchanan-Wollaston et al., 2005; Balazadeh et al., 2008b, 2010b). A regulatory role in developmental senescence, however, has so far only been demonstrated for few genes, including *AtNAP* (At1g69490) and *ORESARA1* (*ORE1*; also called *ANAC092* and *AtNAC2*; At5g39610). Both genes trigger early senescence when overexpressed, while

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a functional block delays senescence (Guo and Gan, 2006; Kim et al., 2009; Balazadeh et al., 2010a), constituting them as positive senescence regulators. As the downstream regulons controlled by senescence-associated NAC TFs are poorly understood, we recently performed microarray-based expression profiling using estradiol-inducible *ORE1* overexpression lines. We found that 78 (~46%) of the 170 genes up-regulated upon *ORE1* induction are known SAGs, suggesting the NAC factor exerts its senescence control function through control of many known senescence-regulated genes (Balazadeh et al., 2010a). Global expression profiling revealed that 36 of the 78 SAGs are induced by long-term (4-d) salt stress, a major promoter of plant senescence, resembling the behavior of *ORE1*, which itself is salt-responsive (He et al., 2005; Balazadeh et al., 2010a, 2010b). Binding sites for ORE1 were found to be present in 26 of the salt-regulated SAGs (Balazadeh et al., 2010b). Additionally, we found that 14 of the 36 salt-triggered SAG genes are induced by hydrogen peroxide (H_2O_2) treatment. Of note, 15 senescence-associated NAC genes (senNACs), including *ORE1*, were found to be H_2O_2-responive (Balazadeh et al., 2010b). It thus appears that salt-triggered senescence at least in part involves H_2O_2-mediated signaling through NAC TFs.

Senescence- and salt-dependent *ORE1*/*ANAC092* expression is controlled through transcriptional regulation, as demonstrated by promoter–reporter gene fusions (Balazadeh et al., 2010a); however, physiologically relevant upstream transcription factors controlling *ORE1* expression during senescence are unknown. *ORE1* expression is under control of the ethylene signaling pathway and is subject to regulation by *miRNA164* (He et al., 2005; Kim et al., 2009).

To identify additional regulators of senescence, we screened T-DNA insertion lines for NAC genes and found that *ORS1* (*ORE-SARA1 SISTER1*; At3g29035) positively controls leaf senescence. *In vitro* binding site selection identified DNA sequence motifs recognized by ORS1 transcription factor. Members of the ORS1 downstream regulon were identified by expression profiling after chemical induction of *ORS1* overexpression. Finally, we demonstrate evolutionary conservation of *ORS1*- and *ORE1*-orthologous promoters in other species of the Brassicaceae family.

RESULTS

ORS1 Defines a Novel Positive Senescence Regulator in *Arabidopsis*

To disclose novel regulators of senescence, we screened available T-DNA insertion lines for NAC genes and found that a mutant carrying a T-DNA in gene At3g29035 (GABI-Kat line 778C04) is late-senescent (see below). According to Ooka et al. (2003), At3g29035 is a paralog to *ORE1*; thus, to indicate its phylogenetic and functional relationship with *ORE1*, we named it *ORS1* for *ORE1 SISTER1*. Within their NAM domains, ORE1 and ORS1 proteins share an overall amino acid identity of 94%; sequence identity amounts to around 41% in the C-terminal part of the two proteins. ORS1 and ORE1 share only limited sequence similarity with AtNAP, namely ~ 63% amino acid identity within the NAM domain and below 23% in the C-terminal region (not shown).

The *ORS1* gene harbors three exons and encodes a protein of 318 amino acids. The *ors1-1* mutant carries the T-DNA in the third exon of the NAC gene (Figure 1A). Absence of functional *ORS1* transcript in fully expanded leaves of homozygous *ors1-1* plant was demonstrated by RT–PCR (Figure 1B). Phenotypic analysis of the null mutant revealed delayed senescence in comparison to the wild-type controls (Figure 1C), which irregularly was accompanied by a small delay (up to 5 d) in flowering time, but no significant change of rosette leaf number when grown under long-day conditions.

Sixty days after sowing, a significantly higher chlorophyll content was observed in the five biggest rosette leaves of the *ors1-1* mutant (Figure 1D), and the percentage of green leaves was approximately three-fold higher in the mutant than the control (data not shown), reflecting a delay in senescence. Accordingly, expression of the senescence marker gene *SAG12* (Weaver et al., 1998) was approximately eight-fold higher in wild-type than *ors1-1* mutant plants (Figure 1E). Furthermore, expression of another senescence-associated gene, *SAG13*, was approximately two-fold higher in the wild-type, as revealed by Affymetrix ATH1 microarray hybridization and qRT–PCR (not shown). We generated a homozygous *ors1-1*/*anac092-1* double mutant but obtained no evidence for a significant further delay in leaf senescence compared to the single-gene mutants under our experimental conditions (not shown). This result indicates that both NAC TFs target regulatory networks that are both important for senescence control.

We tested *ORS1* expression in leaves of early- and late-senescent accessions of *Arabidopsis* (Balazadeh et al., 2008a), 30–60 d after sowing. *ORS1*, like *ORE1*/*ANAC092* (Balazadeh et al., 2010a), was more strongly expressed in early-senescent accessions Col-0 and Lip-0 than in late-senescent accession N13 (Figure 1F), indicating a positive correlation of *ORS1* expression level with the age-dependent senescence status in the different accessions.

We next inhibited *ORS1* by RNA interference (RNAi) controlled by the CaMV 35S promoter; qRT–PCR revealed many lines with almost undetectable *ORS1* transcript (not shown). No senescence was visible in leaves of RNAi lines 40 d after sowing (DAS), in contrast to empty vector (EV) transformed control plants (Figure 1G). Chlorophyll content in leaves no. 7 and 8 was significantly higher in *ORS1* RNAi than in EV lines at 40DAS and the number of fully viable leaves (without visible marks of senescence) was bigger in RNAi than in control plants (not shown). This observation was consistent with low *SAG12* expression in RNAi lines (not shown).

During the course of more than 3 years, we regularly cultivated *anac092-1* (Balazadeh et al., 2010a) and *ors1-1* mutants next to each other and normally found a stronger delay of senescence in the *anac092-1* mutant. However, infrequently, we observed a more pronounced delay of senescence in the *ors1-1* mutant, indicating that unknown environmental factors contribute to determining the contribution of each gene to the

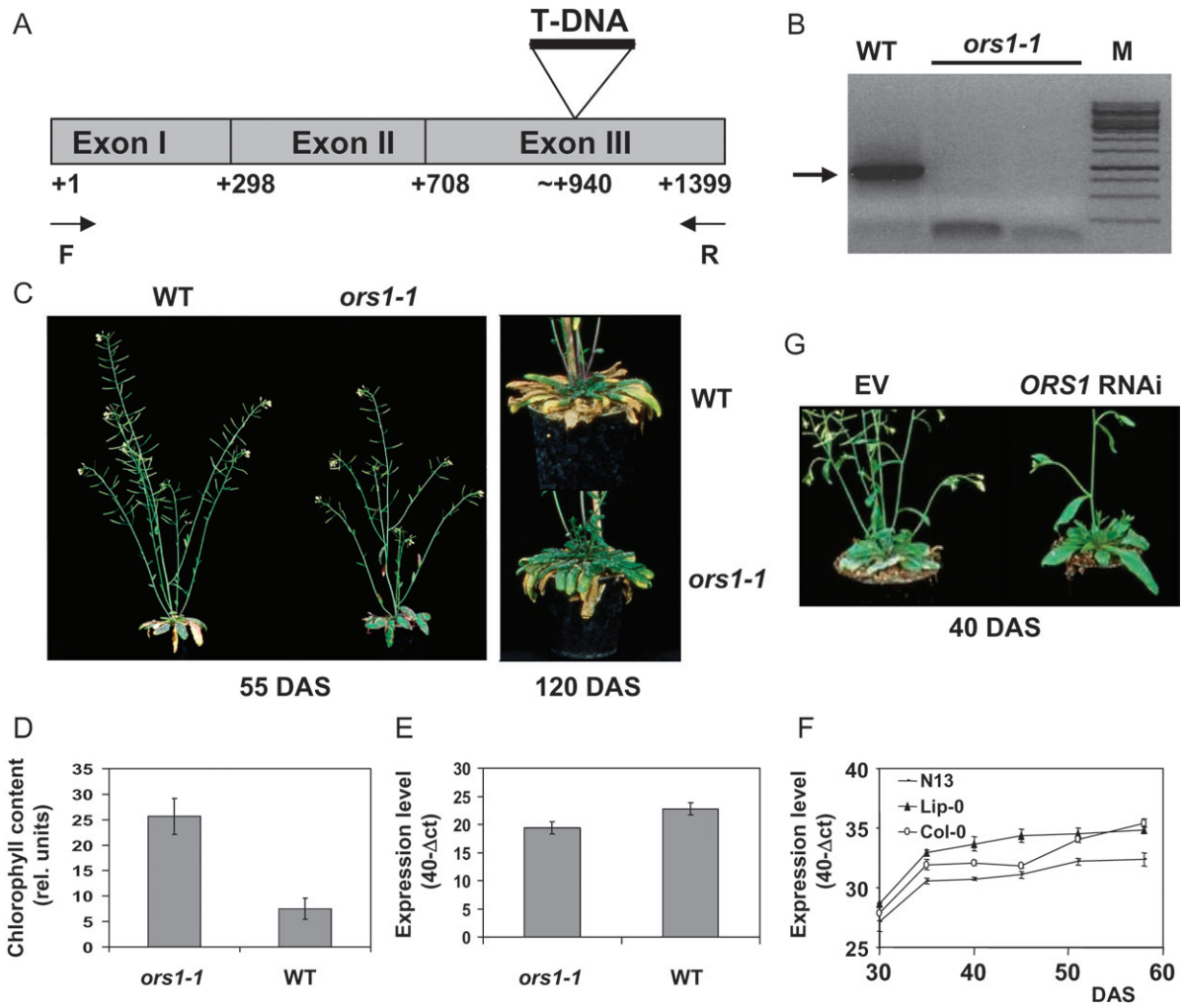

Figure 1. Delayed Senescence in *ors1-1* Mutant and *ORS1* RNAi Lines.

(A) T-DNA is inserted in exon III of *ORS1*.

(B) Absence of *ORS1* transcript (arrow) in *ors1-1* mutant plants, shown by RT–PCR with primers annealing to the start and stop regions of the coding segment. Forward (F) and reverse (R) primer positions are indicated by arrows in (A). WT, wild-type (Col-0); M, molecular size marker.

(C) *Ors1-1* mutant showing delayed senescence, 55 d after sowing (DAS) at long-day conditions and 120 DAS at short-day conditions.

(D) Chlorophyll content of the five biggest leaves from plants shown in (C).

(E) *SAG12* expression in mutants and wild-type plants shown in (C) determined by qRT–PCR. Note that one cycle difference in the qRT–PCR corresponds to a two-fold difference in gene expression.

(F) *ORS1* expression in the five biggest leaves of early- (Lip-0 and Col-0) and late- (N13) senescent accessions at different days after sowing (DAS), determined by qRT–PCR. The Y-axis indicates 40-ΔC_t, where ΔC_t is equal to $C_{t\ gene\_of\_interest} - C_{t\ reference\_gene\_UBQ10}$. Data are means of three independent experiments ± SD. Differences in expression levels are significant for all comparisons between N13 and Lip-0 or Col-0, respectively (Student's *t*-test, $p < 0.05$) with the following exception: 30 DAS, N13 vs. Col-0.

(G) Delayed senescence in *ORS1* RNAi line, 40 DAS.

senescence control network. We also noticed a stronger delay of senescence in *ORS1* and *ORE1* RNAi lines, compared to their respective T-DNA insertion mutants, indicating that the RNAi constructs inhibited not only their cognate genes, but also some other, sequence-related NAC genes.

ORS1 Overexpression Promotes Early Leaf Senescence

We next overexpressed *ORS1* in transgenic *Arabidopsis* plants, confirmed by Northern blot analysis (examples shown in Figure 2A) and qRT–PCR (not shown). Under long-day conditions, *35S:ORS1* lines developed senescence much earlier than wild-type or EV control lines (Figure 2B). We determined the chlorophyll content of the six first emerging leaves (i.e. leaves no. 1–6) of *35S:ORS1* transformants at 35 DAS. As seen in Figure 2C, chlorophyll content of the oldest rosette leaves (leaves no. 1–4) was considerably lower in *35S:ORS1* overexpressor than EV lines. Chlorophyll content was only slightly reduced in leaf no. 5, and almost unchanged relative to control in leaf no. 6, which, under our experimental conditions, was the youngest leaf of the rosette. Thus, overexpression of

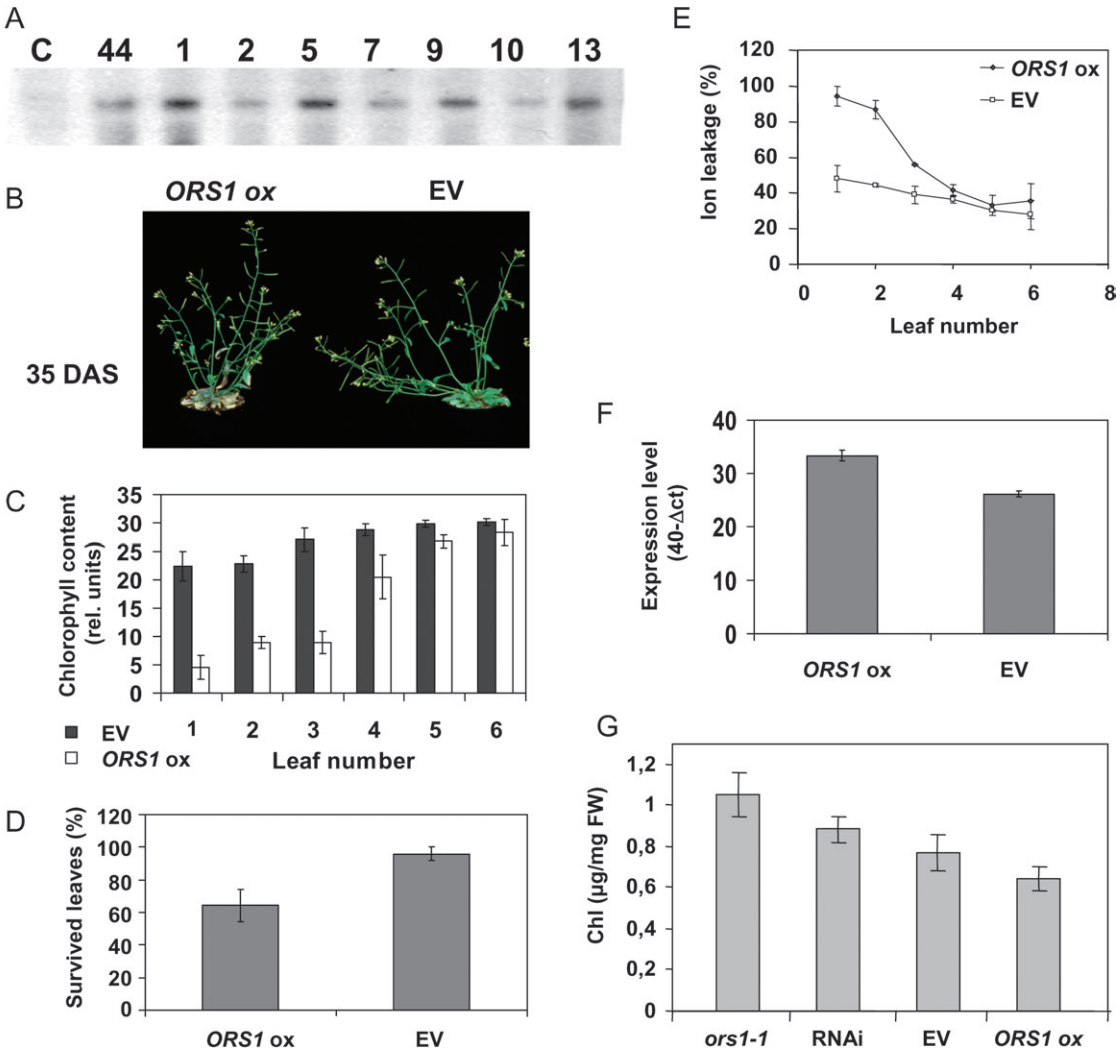

Figure 2. *ORS1* Overexpression Plants.

(A) Northern blot analysis of plants transformed with the *35S:ORS1* construct. Numbers indicate individual transformants. C, control (untransformed) plant. Blots were hybridized with ^{32}P-labeled *ORS1* cDNA probe.

(B) Early senescence in *ORS1* overexpression lines at 35 DAS compared to an empty vector (EV) control plant.

(C) Chlorophyll content of the first six leaves of *35S:ORS1* plants in comparison to EV lines.

(D) Percentage of survived leaves.

(E) Ion leakage.

(F) *SAG12* expression in *35S:ORS1* and EV lines, determined by qRT–PCR. Data in (C)–(F) were obtained from plants at 35 DAS; means ± SD of at least three replicates. Plants were grown under long-day conditions (16 h/8 h, light/dark).

(G) Chlorophyll content in rosette leaves of *ors1-1*, *ORS1–RNAi*, EV, and *35S:ORS1* lines. Leaves were detached from 5-week-old plants, placed on moist filter paper in Petri dishes, and kept in the dark for 4 d. Means ± SD of three replicates. Chlorophyll levels in *ors1-1* versus EV and *35S:ORS1* lines were significantly different (Student's *t*-test, $p < 0.05$).

ORS1 does not abolish chlorophyll accumulation in juvenile leaves, whereas it stimulates a more rapid decline in chlorophyll content in older leaves, concomitant with a reduced number of survived leaves at 35DAS (Figure 2D). Senescence is typically accompanied by membrane disintegration and thus ion leakage (e.g. Woo et al., 2001). Figure 2E shows that ion leakage was low and similar in young leaves (leaves no. 5 and 6) of EV and *ORS1* transgenic lines, whereas it was much more pronounced in older leaves of overexpressors than controls. As *ORS1* overexpression did neither affect chlorophyll accumulation nor ion leakage in developing (young) leaves, it appears that full execution of *ORS1* function requires additional, yet unknown factors that are only present in mature leaves. A similar phenomenon exists in *ORE1* overexpression plants (Balazadeh et al., 2010a). As a further indicator for leaf senescence, we determined *SAG12* expression, which was strongly elevated (>30-fold) in shoots of *35S:ORS1* overexpression lines compared to controls (Figure 2F). We also observed that dark-induced senescence developed faster in *35S:ORS1* overexpressors compared to control lines, whereas it was

delayed in the *ors1-1* mutant (and RNAi lines, Figure 2G). Taken together, *ORS1* constitutes an important element of the cellular networks controlling developmental as well as dark-induced senescence. Consistent with this conclusion is the fact that *ORS1* transcript abundance increased strongly (more than eight-fold) under extended night conditions, similarly to *ORE1* (Usadel et al., 2008; GENEVESTIGATOR). Of note, we observed that senescence was generally more pronounced in *35S-ANAC092* (Balazadeh et al., 2010a) than in *35S:ORS1* overexpression lines (not shown), indicating a more prominent role of *ORE1* in senescence control, at least under standard greenhouse growth conditions.

ORS1 Expression Pattern

To investigate *ORS1* expression, we fused its ~1.5-kb-long 5′ upstream regulatory region to β-glucuronidase (*GUS*) reporter gene and tested GUS activity in *Arabidopsis* plants transformed with the $Prom_{ORS1}$*:GUS* construct. Representative expression patterns are shown in Figure 3.

In young seedlings up to an age of ~10 d, strong GUS activity was observed in cotyledons and regularly in the tip regions of very young leaves (Figure 3A(a) and 3A(b)), but not in slightly further developed, green leaves (Figure 3A(b) and 3A(c)). Strong GUS activity was observed in older leaf parts when senescence became apparent (Figure 3A(c)), in agreement with elevated *ORS1* (*ANAC059*) transcript abundance in senescing leaves (Balazadeh et al., 2008b) and rosettes (Buchanan-Wollaston et al., 2005; see GENEVESTIGATOR).

GUS activity was also detected in floral parts, with a preference for old sepals, petals, old stamens, mature anthers, and pollen grains, while immature floral tissue showed no GUS activity (Figure 3A(d), 3A(e), and 3A(g), and data not shown). Strictly localized GUS activity was observed at the floral organ abscission zone of mature flowers (Figure 3A(e)). Maturation of reproductive floral organs and abscission of floral organs are considered to be senescence processes (Bleecker and Patterson, 1997). *ORS1* promoter-driven GUS activity was also observed in roots (Figure 3A(f)).

We also analyzed the transcriptional regulation of *ORS1* in transgenic tobacco and observed significant GUS staining only in older leaf parts (i.e. leaf tips), consistent with an age-dependent regulation of *ORS1* (Figure 3B(a)). Expression of *ORS1* was also wound-inducible in $Prom_{ORS1}$*:GUS* tobacco leaves (not shown). GUS staining was virtually absent from young flowers (Figure 3B(b)), but strong expression was detected in petals of older (opened) flowers (Figure 3B(c)). Similarly, GUS activity was detected in mature, but not immature anthers (Figure 3B(d)), and in roots (Figure 3B(e)).

Binding-Site Selection Defines a Consensus Target Sequence of ORS1

As *cis*-elements recognized by ORS1 were not reported previously, we performed an *in vitro* binding site selection experiment to discover sequence motifs preferred by ORS1 using the CELD–transcription factor fusion method (Xue, 2002, 2005). ORS1 was translationally fused to the catalytic domain of a 6xHis-tagged cellulase D (CELD) from *Neocallimastix patricairum* and incubated with biotin-labeled random-sequence oligonucleotide probes. Oligonucleotides bound by the ORS1–CELD fusion protein were recovered by means of affinity purification of the DNA–ORS1–CELD complex, and the catalytic activity of CELD was used for quantification of the amount of protein bound to the oligonucleotides (Xue, 2002, 2005). Fifteen clones representing 13 unique sequences were obtained and analyzed for binding activity. An alignment of the target sequences and relative binding activities are shown in Table 1. ORS1 binds to a bipartite DNA element of 17–18 bp containing the consensus sequence [AG]CGT[AG](4-5n)[AG][C-T]ACGCAA. The target site thus includes two core motifs, RCGTR (motif 1) and RYACGCAA (motif 2; R = A or G; Y = C or T), separated by a spacer of 4–5 bp. Nucleotide substitution experiments performed on the basis of oligonucleotide 3 indicated the relevance of some, but not all nucleotide positions for efficient ORS1 binding (Table 1; oligonucleotides 3m1 to 3m4). We noticed a dramatic drop in ORS1 binding activity upon reducing the distance between the two core motifs from 4 to 3 bp, or increasing it to 6 bp (oligonucleotides 3m5 and 3m7), whereas increasing it from 4 to 5 bp did not affect binding activity (oligonucleotide 3m6). Thus, ORS1 has highest affinity to target DNA when the two core motifs are spaced by 4 or 5 bp. The bipartite recognition site occurs in a number of genes controlled by ORS1 in dexamethasone-inducible overexpression lines (see below).

The ORS1 Regulatory Network

To identify genes downstream of ORS1, we expressed it under the control of a chemically (dexamethasone, DEX) inducible system in *Arabidopsis*, and studied global transcriptome changes using Affymetrix ATH1 microarrays 5 h after DEX treatment. In the transgenic *ORS1–DEX* plants, ORS1 is genetically fused to a glucocorticoid receptor (GR). As expression of the chimeric gene is controlled by the CaMV 35S promoter, its expression level remains constant before and after induction, and DEX treatment induces the nuclear targeting of the fusion protein (Lloyd et al., 1994). To filter out possible effects induced by DEX application rather than overexpression of *ORS1*, we performed the same experiment with ethanol-treated *ORS1–DEX* plants. To find downstream target genes of ORS1, we chose 46-day-old plants when most leaves were mature, shortly before senescence became visible. *ORS1–DEX* plants were sprayed with 30 μM DEX or 0.5% ethanol (controls). RNA isolated from whole rosettes was subjected to expression profiling.

This experiment, performed in two biological replications, yielded 42 up-regulated and 26 down-regulated transcripts (considering absolute $\log_2 \leqslant -1.58$ and $\geqslant 1.58$) (Table 2 and Supplemental Table 1). Among the up-regulated genes, 30 transcripts (~70%) were previously shown to be up-regulated during senescence (e.g. Guo et al., 2004; Buchanan-Wollaston

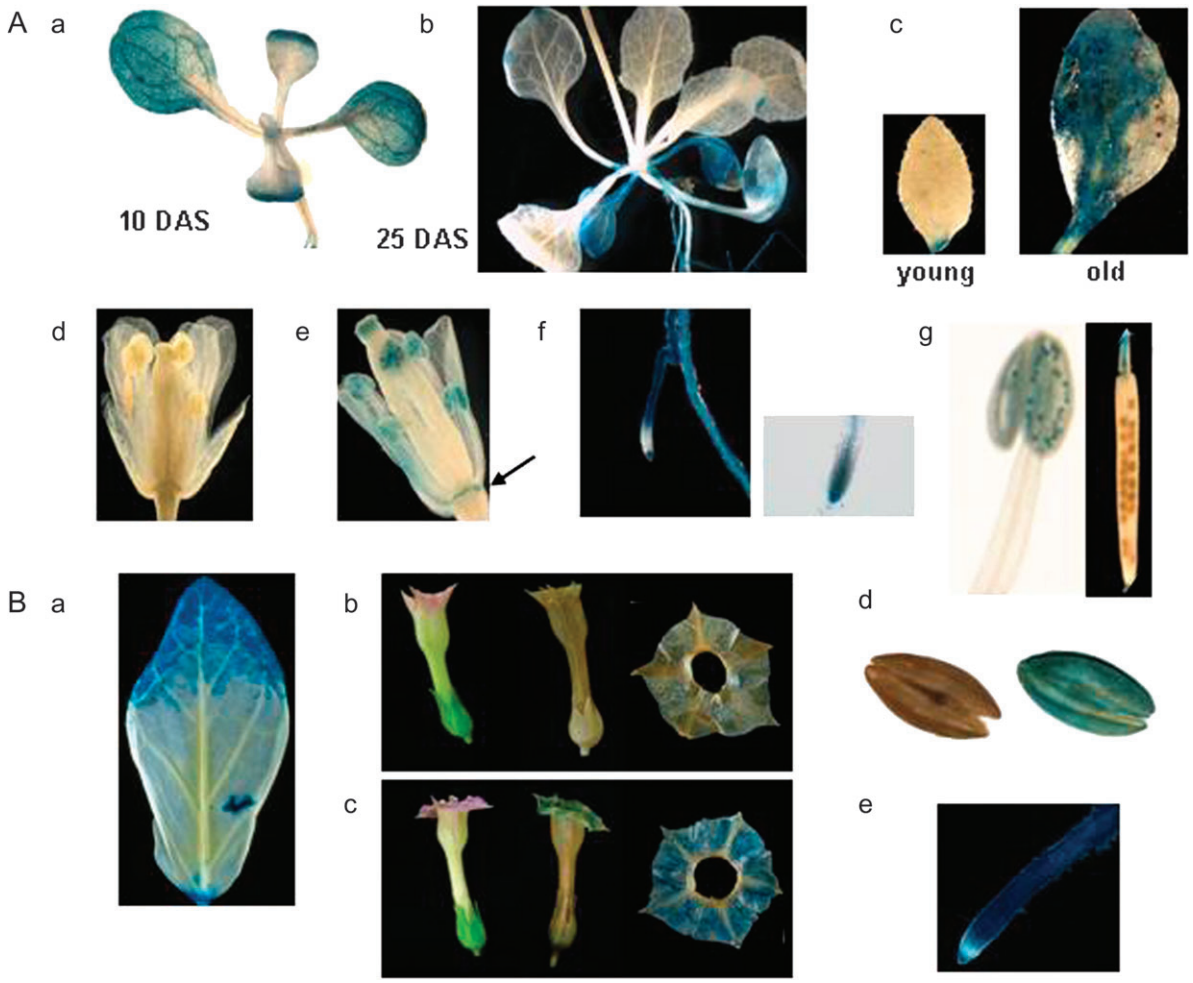

Figure 3. *ORS1* Promoter-Driven *GUS* Expression.

(A) *Arabidopsis*. (a) GUS expression in 10-day-old seedling. (b) Leaves of a 25-day-old seedling. Note GUS staining in the leaf tip. (c) GUS staining in young and old leaves from a soil-grown ~5-week-old plant. (d) Immature flower. (e) Mature flower with GUS staining in stamens and the floral abscission zone (arrow). (f) Roots. (g) Anther and mature silique.

(B) Tobacco. (a) Leaf with GUS staining in the tip region. (b) Immature flower. (c) Mature flower with GUS staining in petals. (d) Young (left) and mature (right) stamens. (e) Root. Incubation time in GUS staining solution was less than 1 h in all cases.

et al., 2005; van der Graaff et al., 2006; Balazadeh et al., 2008b). This result is in accordance with the model that ORS1 is a senescence-regulatory transcription factor, although it appears to regulate fewer genes than ORE1/ANAC092. As previously reported, 170 genes were at least two-fold up-regulated 5 h after induction of *ORE1* (Balazadeh et al., 2010a). Comparing the two datasets revealed that only eight genes were commonly up-regulated (Table 2), indicating that the two NAC TFs control gene sets that only partly overlap. This observation is in accordance with the fact that ORE1 binds to the core binding site of the ORS1 factor, but more flexibly tolerates single-nucleotide mutations in the second motif of the NAC binding site while basically retaining its binding activity (data not shown). We also found that induction of *ORS1* at the seedling stage only affected few SAGs (not shown), indicating that additional factors required for senescence-induced gene expression are missing in young tissues.

We selected 13 of the 42 up-regulated genes (Table 2) and tested their DEX-dependent expression in a third biological replicate by quantitative real-time PCR. DEX-triggered enhancement of gene expression was confirmed in all cases (Table 2). Over-representation analysis using PageMan (Usadel et al., 2006) revealed a significant enrichment of detoxification (e.g. glutathione-S transferases, *P*-value: 2×10^{-6}) and antioxidant genes (e.g. glutathione redoxins, *P*-value: 0.002) among the up-regulated transcripts (not shown).

We next searched for the presence of the ORS1 binding sites in the 1-kb upstream regions of DEX-responsible genes in *ORS1–DEX* plants using the MGT(N_{7-8})ACGY core derived from the ORS1 binding site selection and mutation studies. The core sequence allows for at least 30% binding activity compared to sequences primarily selected by the CELD assay and may thus contribute to regulation by ORS1 *in vivo*. Of the 42 genes whose expression was up-regulated upon *ORS1* induction,

Table 1. Binding Site Selection.

Oligonucleotide	ORS1-selected oligonucleotides	Relative binding activity (%)
1	AACACACACATG**ACGTA** ATAC **ACACGCAA**Cc	100 ± 1.2
2	TCGATT**GCGTA** CACGT **ACACGCAA**CCTACC	93 ± 1.2
3	CGGGGTT**ACGTA** CGGC **ACACGCAA**CCGTGC	93 ± 1.1
4	TCGAGTT**GCGTG** ACGG **ACACGCAA**CCTACC	85 ± 1.3
5	AA**ACGTA** AACC **ACACGCAA**TGTAGAGCCGC	80 ± 0.7
6	TA**GCGTA** CGTTT **ACACGCAA**GCTACTTGTA	78 ± 0.6
7	a**gcGTG** CCTT **ACACGCAA**CCGTTGCCGGTTCGA	75 ± 0.3
8	ACCGGGTA**ACGTA** TACA **GCACGCAA**ACCTA	74 ± 0.5
9	a**gcGTG** TGGA **GTACGCAA**TGTCATTGTATACCC	74 ± 0.4
10	a**gcGTG** TTGC **ATACGCAA**CCTCGCACTGCTTAC	71 ± 0.2
11	a**gcGTG** TGGT **ACACGCAA**CTGAGTGTGTGCCTG	65 ± 0.6
12	a**gcGTG** TGCC **GTACGCAA**TCCAGTCCCTCCTCG	64 ± 1.4
13	a**gcGTA** CAGT **TCACGCCA**CCGCACCCATCTCCA	52 ± 1.1
Consensus	**RCGTR** N(4-5)**RYACGCAA**	
ANAC019, 55, 72	**TC**NNNNNNN**ACACGCATGT**	
TaNAC69	**CGTR** NNNNN **YACG**	
	Mutated oligonucleotides (substitutions)	
3	CGGGGT**TACGTA** CGGC **ACACGCAACC**GTGC	100 ± 0.7
3m1	CGGGGT**TACGTA** CGGC **ACAC<u>A</u>CAACC**GTGC	29 ± 0.2
3m2	CGGGGT**TA<u>A</u>GTA** CGGC **ACACGCAACC**GTGC	52 ± 0.5
3m3	CGGGGTT**<u>G</u>CGTA** CGGC **ACACGCAACC**GTGC	113 ± 0.2
3m4	CGGGGT**TACGTA** CGGC **ACACG<u>T</u>AACC**GTGC	108 ± 0.8
	Mutated oligonucleotides (additions and deletions)	
3m5	CGGGGT**TACGTA** GGC **ACACGCAACC**GTGC	4 ± 0.1
3m6	CGGGGT**TACGTA<u>C</u>**CGGC **ACACGCAACC**GTGC	109 ± 0.2
3m7	CGGGGT**TACGTA<u>CT</u>**CGGC **ACACGCAACC**GTGC	11 ± 0.2

Sequence alignment of ORS1-selected oligonucleotides 1–13. Binding activity of ORS1 to oligonucleotide 1 was set to 100%. Values are means ± SD of three assays. Lower-case letters are from flanking primer sequences. Binding sites for ANAC019, 55 and 72 from *Arabidopsis* (Tran et al., 2004) as well as for TaNAC69 from wheat (Xue, 2005) are indicated for comparison. Oligonucleotides 3m1, 2, 3, and 4 were derived from oligonucleotide 3 by base substitution within the consensus regions. Oligonucleotides 3m5, 6, and 7 were derived from the same oligonucleotide by the deletion or addition of nucleotides in the linker sequence (changed bases underlined). Binding activities for mutated oligonucleotides are given relative to that of oligonucleotide 3.

21 harbor at least one ORS1 binding site within their 1-kb promoter (Supplemental Table 2).

Salt- and Hydrogen Peroxide-Dependent *ORS1* Expression

We have previously shown that salt stress triggers the expression of many genes that are downstream of ORE1/ANAC092, indicating that this transcription factor plays a role in salt-induced senescence (Balazadeh et al., 2010a, 2010b). We therefore tested gene expression in shoots of 28-day-old plants grown in hydroponic condition as described previously (Balazadeh et al., 2010a). Salt stress (150 mM NaCl) was applied to the growth medium for 6 h (short-term stress) and 4 d (long-term stress), respectively. Expression profiling using Affymetrix ATH1 arrays showed that 32 of the 42 DEX-dependent genes (i.e. 76%) observed in *ORS1–DEX* plants were induced by long-term, but not short-term salinity stress (Supplemental Table 3).

Environmental stresses including desiccation and salinity perturb cellular redox state and trigger accumulation of reactive oxygen species (ROS) such as singlet oxygen, superoxide anion radical, hydroxyl radical and hydrogen peroxide (H_2O_2) in plant cells (Miller et al., 2009). Several senescence-regulated NAC genes are also induced by external application of H_2O_2 or treatments that trigger the accumulation of ROS, such as ozone and methyl viologen, or 3-aminotriazole, which blocks catalase leading to a rise in H_2O_2 level (e.g. Davletova et al., 2005; Gechev and Hille, 2005; Gadjev et al., 2006; Balazadeh et al., 2010b). We previously observed that *ORS1* transcript abundance increased approximately two-fold after 1 h H_2O_2 treatment, and approximately five-fold after 5 h, whereas *ORE1* was not induced after 1 h, and induced two-fold after 5 h (Balazadeh et al., 2010b). Here, taking advantage of the *Prom_{ORS1}:GUS* lines, we found a rapid and strong H_2O_2-

Table 2. ORS1-Dependent Up-Regulated Genes.

Affy ID	AGI Code	Annotation	ORS1–DEX 1st	ORS1–DEX 2nd
245148_at	AT2G45220*	Pectinesterase family protein	1.68	2.74
245392_at	AT4G15680	Glutaredoxin family protein	3.46	2.87
245393_at	AT4G16260*	Glycosyl hydrolase family 17 protein	2.01	2.06
245506_at	AT4G15700	Glutaredoxin family protein	2.63	2.78
245976_at	AT5G13080*	WRKY75 (WRKY DNA-binding protein 75)	1.96	2.32
247327_at	AT5G64120*	Peroxidase, putative	1.87	2.54
247925_at	AT5G57560*	TCH4 (TOUCH 4); hydrolase, acting on glycosyl bonds	2.03	1.81
251293_at	AT3G61930*#	Unknown protein	1.67	1.82
252265_at	AT3G49620	DIN11 (DARK INDUCIBLE 11); oxidoreductase	2.67	2.54
252367_at	AT3G48360	BT2 (BTB and TAZ domain protein 2)	2.12	2.04
253161_at	AT4G35770	SEN1 (DARK INDUCIBLE 1)	2.94	2.48
253915_at	AT4G27280*#	Calcium-binding EF hand family protein	2.54	1.63
254042_at	AT4G25810*	XTR6 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 6)	2.08	2.74
254387_at	AT4G21850*	Methionine sulfoxide reductase domain-containing protein/SelR domain-containing protein	2.57	2.30
254889_at	AT4G11650*	ATOSM34 (OSMOTIN 34)	2.66	2.49
255543_at	AT4G01870*	TolB protein-related	2.41	2.56
256012_at	AT1G19250*	FMO1 (FLAVIN-DEPENDENT MONOOXYGENASE 1)	2.65	3.34
256252_at	AT3G11340	UDP-glucoronosyl/UDP-glucosyl transferase family	3.40	3.50
256891_at	AT3G19030*	Similar to unknown protein (*Arabidopsis thaliana*) (TAIR:AT1G49500.1)	3.24	1.70
256933_at	AT3G22600*	Protease inhibitor/seed storage/lipid transfer protein (LTP)	2.11	1.83
257540_at	AT3G21520*	Similar to unknown protein (*Arabidopsis thaliana*) (TAIR:AT3G21550.1)	1.61	2.37
257774_at	AT3G29250*#	Oxidoreductase	2.25	2.82
258203_at	AT3G13950*	Similar to unknown protein (*Arabidopsis thaliana*) (TAIR:AT4G13266.1)	2.34	1.93
258957_at	AT3G01420*	ALPHA-DOX1 (ALPHA-DIOXYGENASE 1)	1.69	2.44
260225_at	AT1G74590*	ATGSTU10 (*Arabidopsis thaliana* Glutathione S-transferase (class tau) 10)	2.03	2.05
260405_at	AT1G69930*	ATGSTU11 (*Arabidopsis thaliana* Glutathione S-transferase (class tau) 11)	2.49	2.37
260522_x_at	AT2G41730	Similar to unknown protein (*Arabidopsis thaliana*) (TAIR:AT5G24640.1)	2.43	1.93
260706_at	AT1G32350*	AOX1D (ALTERNATIVE OXIDASE 1D); alternative oxidase	2.46	3.47
261135_at	AT1G19610*	LCR78/PDF1.4 (low-molecular-weight cysteine-rich 78)	4.10	2.40
261763_at	AT1G15520*	ATPDR12/PDR12 (PLEIOTROPIC DRUG RESISTANCE 12)	3.21	3.70
261892_at	AT1G80840*	WRKY40 (WRKY DNA-binding protein 40)	2.26	2.74
262085_at	AT1G56060	Similar to unknown protein (*Arabidopsis thaliana*) (TAIR:AT2G32190.1)	2.67	2.53
262399_at	AT1G49500	Similar to unknown protein (*Arabidopsis thaliana*) (TAIR:AT3G19030.1)	3.26	1.78
263948_at	AT2G35980*	YLS9 (YELLOW-LEAF-SPECIFIC GENE 9)	3.70	4.05
264016_at	AT2G21220	Auxin-responsive protein, putative	1.76	1.61
265658_at	AT2G13810*	ALD1 (AGD2-LIKE DEFENSE RESPONSE PROTEIN1)	2.63	2.24
266267_at	AT2G29460*	ATGSTU4 (GLUTATHIONE S-TRANSFERASE 22)	2.73	1.72
266270_at	AT2G29470*	ATGSTU3 (GLUTATHIONE S-TRANSFERASE 21)	1.90	3.42
266658_at	AT2G25735	Unknown protein	2.50	1.95
267147_at	AT2G38240*	Oxidoreductase, 2OG-Fe(II) oxygenase family protein	2.48	2.33
267385_at	AT2G44380	DC1 domain-containing protein	1.89	2.18
267567_at	AT2G30770*	CYP71A13 (cytochrome P450, family 71, subfamily A, polypeptide 13); oxygen binding	2.28	2.90

Numbers in the two right-most columns indicate expression values in two biological replicates, given as fold change on log2 basis (Dex treatment compared to mock).

***** Senescence-massociated genes. Genes tested by qRT–PCR for expression in the 3rd biological replicate are underlined. **#** Genes up-regulated at least two-fold upon estradiol-mediated induction of *ORE1/ANAC092* expression in *ANAC092-IOE* plants (Balazadeh et al., 2010a). If a two-fold induction threshold is also considered for genes responding to *ORS1* induction in *ORS1–DEX* plants (this report), five more genes overlapped with the *ANAC092-IOE* dataset: At3g01830, At3g61190, At2g32680, At5g38710, and At5g39520.

triggered induction in GUS signal already after 1h (Figure 4A and 4B). Elevated GUS staining was observed in both roots and leaves; however, a quicker and stronger response to H_2O_2 was observed in roots (not shown). Next, we tested the effect of H_2O_2 on a series of transgenic plants harboring successive *ORS1* promoter deletions fused to *GUS* (see below) and observed significantly reduced H_2O_2-induced GUS staining in plants carrying –204 or –161 deletions (examples shown in Figure 4A). Additionally, we quantitatively determined *ORS1* promoter activity by 4-methylumbelliferyl-beta-D-glucuronide (4-MUG) assay. As shown in Figure 4B, reporter gene activity in *Arabidopsis* seedlings was strongly enhanced after 1 h of H_2O_2 treatment (10 mM) in plants carrying the full-length *ORS1* promoter. High H_2O_2-dependent promoter activity was retained in deletions down to –230 bp, but was lost upon further deletion (Figure 4B), indicating that regulatory elements controlling H_2O_2-triggered expression of *ORS1* are located within the proximal 230-bp promoter region, but are absent in the –204-bp deletion. We also tested H_2O_2-dependent *ORE1/ANAC092* transcriptional activation using $Prom_{ANAC092}$*:GUS* seedlings (Balazadeh et al., 2010a). In contrast to *ORS1*, enhanced GUS staining was only observed after 5 h in $Prom_{ANAC092}$*:GUS* lines; no difference in staining was observed after 1 h of H_2O_2 treatment, indicating a more delayed response, consistent with expression changes of the two genes as determined by qRT–PCR (see above).

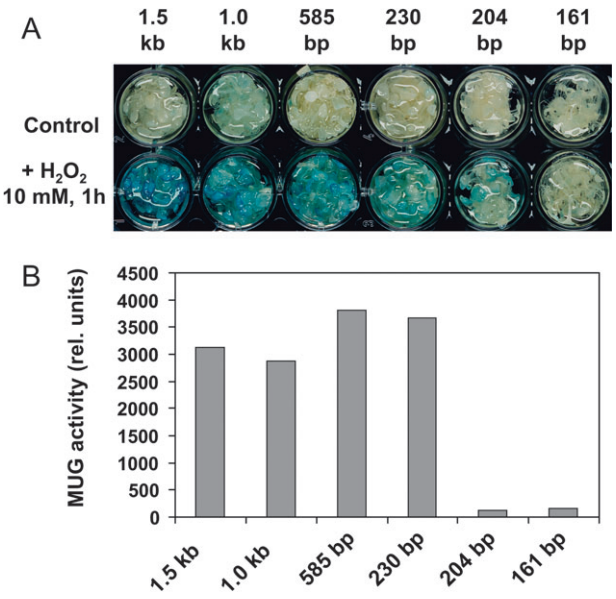

Figure 4. H_2O_2-Dependent *ORS1* Expression.

GUS activity in 2-week-old *Arabidopsis* seedlings transformed with $Prom_{ORS1}$*:GUS* and $Prom_{ORS1del}$*: GUS* constructs, treated for 1 h with 10 mM H_2O_2 compared to control.

(A) Histochemical assay. GUS staining was performed for 30 min.

(B) 4-Methylumbelliferyl-beta-D-glucuronide (4-MUG) assay. MUG activity is given as relative value, where the activity of H_2O_2-treated plants was compared to control condition.

We then analyzed H_2O_2-dependent expression of ORS1 downstream genes and found that 24 of those were significantly induced after 5 h H_2O_2 treatment; 16 were already induced after 1 h incubation time (Supplemental Table 3). We conclude that ORS1, similar to ORE1, triggers expression of SAGs; part of this regulatory network appears to involve cross-talk with salt- and H_2O_2-dependent signaling pathways.

ORS1 and *ORE1* Are Concertedly Evolving Genes with an Ancient Evolutionary Origin

Phylogenetic analysis of NAC domains indicated that ORS1 and ORE1 transcription factors are closely related members of the NAC family in *Arabidopsis* (Ooka et al., 2003). To test evolutionary conservation of *ORS1* and *ORE1* genes, we screened for orthologous genes in selected species of the Brassicaceae family. Orthologous versus paralogous relationships of homologous genes can be difficult to discriminate, especially in the case of multigene families. Thus, to avoid potential mis-assignments, we cloned upstream regulatory regions, instead of the coding regions, of *ORS1* and *ORE1* homologous, and included the 5′ untranslated regions as specific, highly conserved marker segments. The *Arabidopsis ORS1* and *ORE1* 1-kb upstream regions (counted from the translation initiation codon) were run as BLAST baits against sequences deposited in the *Brassica oleracea* Genome Project Database (www.tigr.org/tdb/e2k1/bog1/). Brassica and *Arabidopsis* separated approximately 12–24 million years ago (Yang et al., 1999). A PCR-based approach was used to clone *ORS1* and *ORE1* 5′ upstream regions from *Arabidopsis arenosa*, *Brassica oleracea* cv. Capitata alba, *Capsella rubella*, and *Raphanus sativus* cv. Nancy, respectively. Phylogenetic footprinting analysis using ConSite (Sandelin et al., 2004) indicated significant conservation of *ORS1* and *ORE1* 5′ upstream regions for all species. The *ORE1* promoter from *A. thaliana* exhibited highest sequence similarity, defined through an automatically calculated value (Sandelin et al., 2004), with the *A. arenosa ORE1* promoter, whereas the lowest conservation level was observed when compared with the *B. oleracea* ortholog (Supplemental Figure 1A). Notably, promoter conservation across species was above 90% in all pair-wise comparisons of *ORE1* orthologs, suggesting that most segments of the promoter are under positive selection. The overall conservation level was significantly lower for the *ORS1* ortholog, ranging from 72 to 94% (Supplemental Figure 1B), suggesting a lower selective pressure for conservation of the *ORS1* promoter when compared with the *ORE1* promoter. Nonetheless, the observed conservation level is significant and presumably reflects a regulatory role of this region. Moreover, significant conservation (70%) was also observed between promoters of *ORS1* and *ORE1* in *Arabidopsis*. These findings were supported by further analyses using the FootPrinter motif discovery tool (Blanchette and Tompa, 2003). FootPrinter allows the identification of highly conserved non-coding sequences (CNSs) in promoters of orthologous subsets with a higher resolution than ConSite. Testing various parameters (minimal motif sizes; maximum

number of mutations accepted within the motifs) supported the conclusion that *ORE1* promoters were more strongly conserved throughout evolution than *ORS1* promoters. In *ORE1* promoters, FootPrinter revealed a set of six conserved non-coding sequences that were 21 (most distal CNS), 10, 20, 16, 23, and 17 (most proximal CNS) nucleotides long, respectively (Figure 5A). Using identical screening parameters, only four non-mutated motifs of at least 10 nucleotides (i.e. 20, 11, 12, and 10 nucleotides, respectively) were identified in *ORS1* promoters (Figure 5C). With less stringent conditions, FootPrinter discovered more and longer conserved non-coding sequences in both, *ORS1* and *ORE1* orthologous sets. Nevertheless, the overall conservation level of *ORE1* orthologs was significantly higher than of *ORS1* orthologs (Figure 5B and 5D, respectively). We scanned the conserved promoter fragments for the presence of known *cis*-acting regulatory elements, using information from the PLACE database. This analysis revealed six and seven previously described regulatory elements, respectively, in the phylogenetic footprints of *ORS1* and *ORE1* orthologs (Figure 5A and 5C). The majority of these elements were shown to have a function in stress, wound, or salicylic acid responses, which is consistent with the observation that expression of *ORS1* and *ORE1* genes is controlled by these factors (GENEVESTIGATOR).

Identification of a Promoter Region Required for Senescence-Associated Gene Expression

To define regulatory element(s) that control senescence-associated expression, we performed *ORS1* promoter deletions. *Arabidopsis* plants transformed with the $Prom_{ORS1del}$:*GUS* constructs were analyzed for senescence-dependent expression; deletions to positions –1000, –585, –400, and –230 bp did not impair *ORS1* expression in senescent leaves. We then tested for the presence of highly conserved non-coding sequences (CNSs) in the 230-bp promoter region, taking advantage of the sequences of *ORS1* orthologs (see above). A sequence logo created using the WebLogo software (http://weblogo.berkeley.edu/) demonstrated sequence conservation within this part of the *ORS1* promoter (Figure 6A). Gaps in the logo result from insertions/deletions in the aligned promoter sequences. Additional 5′ deletions of the *ORS1* promoter were generated to test the functions of the non-coding sequences. Thus, –204 and –161 promoter constructs were made to delete CNS1 and CNS2, respectively, and transformed into *Arabidopsis*. Senescence-specific expression of *ORS1* was significantly reduced in the –204 deletion. This was also the case for expression in flowers, especially in the abscission zone, while no significant changes in the expression were observed in roots (Figure 6B). The same expression pattern was observed for the –161 deletion (not shown). These results indicate that the promoter region between –230 and –204 contains *cis*-element(s) that are required for senescence-specific expression of *ORS1*. Currently, however, the upstream transcription factors binding to this element to regulate *ORS1* transcription remain unknown.

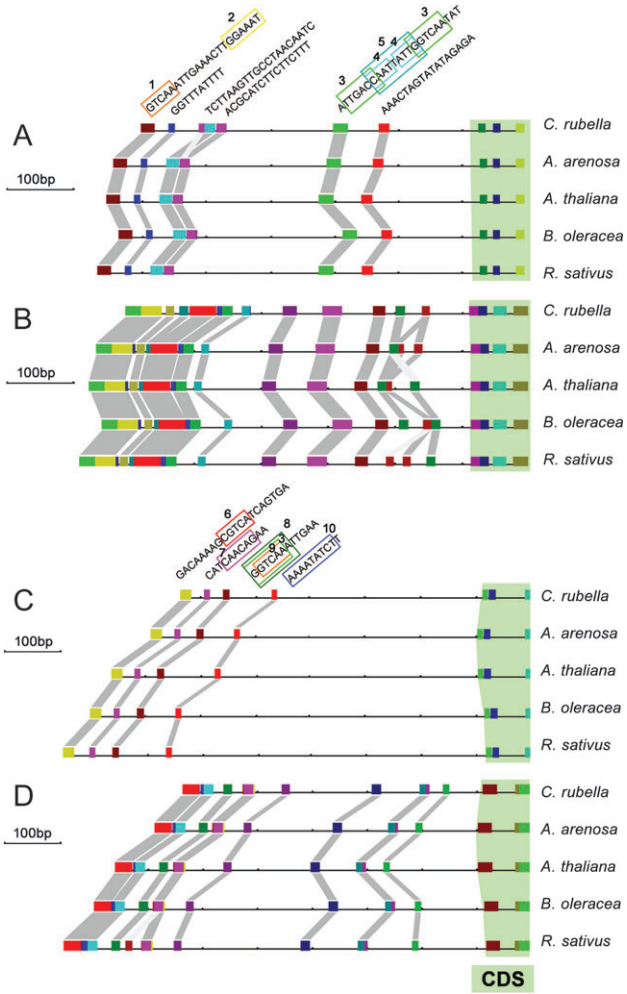

Figure 5. Conserved Non-Coding Sequences in Promoters of *ORS1* and *ORE1* Orthologs.

Promoter sequences were obtained from *Arabidopsis thaliana*, *Arabidopsis arenosa*, *Capsella rubella*, *Raphanus sativus*, and *Brassica oleracea*.

(A) *ORE1* orthologous promoters. Minimal motif size (MMS): 10; maximal number of mutations accepted within the motifs (MNM): 0. Fully conserved sequences in *ORE1* promoters are shown above the alignments. Boxes shown in color indicate known *cis*-acting elements (>4 bp long) deposited in the PLACE database. 1, W box, wounding, salicylic acid and stress response element; 2, GT-1 binding site, light-regulated transcription; 3, WRKY binding site; 4, heat shock response element; 5, HD-Zip protein binding element.

(B) *ORE1* orthologous promoters. MMS: 12; MNM: 2.

(C) *ORS1* orthologous promoters. MMS: 10; MNM: 0. Fully conserved sequences in *ORS1* promoters are indicated. Boxes shown in color indicate known *cis*-acting elements (>4 bp long). 6, ASF-1 binding site, transcriptional activation by auxin and/or salicylic acid; 7, ATMYB2 binding site, water stress response element; 8 and 3, WRKY binding site; 9, W box, wounding, salicylic acid and stress response element; 10, *cis*-elements for ethylene and circadian regulation.

(D) *ORS1* orthologous promoters. MMS: 12; MNM: 2.

Gray lines connect identical conserved non-coding sequences; blocks shown in green highlight coding sequences (CDS) of *ORS1* and *ORE1* orthologs.

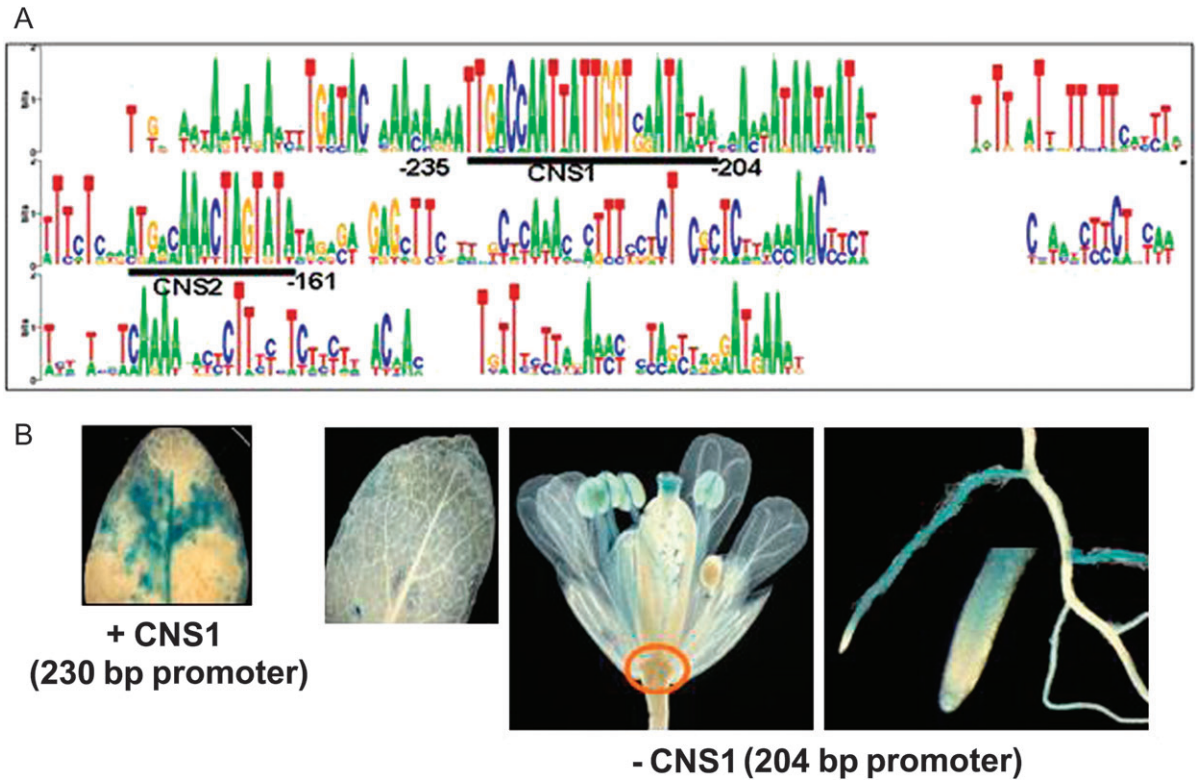

Figure 6. Deletion of Conserved Non-Coding Sequences in the *ORS1* Promoter.

(A) Representation of highly conserved sequences in the proximal part of the *ORS1* promoter using WebLogo software (http://weblogo.berkeley.edu/).

(B) *ORS1*-204 promoter-driven GUS expression. Note the almost complete absence of expression in senescent leaves and the abscission zone of mature flowers (encircled). GUS activity in senescent leaves is still driven by the –230-bp promoter deletion harboring CNS1 (shown on the left) and the flower abscission zone (not shown).

DISCUSSION

Senescence is a multifaceted process that integrates developmental programs with environmental inputs controlled by intricate gene regulatory networks involving an appreciable number of transcriptional and other regulators. Although many NAC TFs have previously been observed to undergo senescence-dependent changes in gene expression (e.g. Buchanan-Wollaston et al., 2005; Gregersen and Holm, 2007; Balazadeh et al., 2008b, 2010b), only a few have firmly been proven to control senescence, including the *Arabidopsis* genes *AtNAP* and *ORE1* (Guo and Gan, 2006; Kim et al., 2009; Balazadeh et al., 2010a). Based on our experimental data presented here, we conclude that *ORS1* constitutes a further positive regulator of senescence that adds to the functions of *ORE1* and *AtNAP*. According to our phylogenetic promoter analysis, *ORS1* and *ORE1* genes originated through an early duplication event, most likely already in the ancestor of the Brassicaceae family. Although the promoters of both genes diverged considerably during subsequent evolution, various conserved non-coding sequences were retained, indicating their functional relevance and that both genes are important for the control of leaf senescence not only in *Arabidopsis thaliana*, but also in other species of the Brassicaceae family.

Downstream targets have previously only been reported for ORE1 (Balazadeh et al., 2010a). Here, we discovered genes that rapidly respond to a change ORS1 nuclear localization, induced by dexamethasone *ORS1–DEX* plants. Notably, the majority (~70%) of the ORS1 up-regulated genes were previously reported to be induced during senescence (e.g. Guo et al., 2004; Buchanan-Wollaston et al., 2005; van der Graaff et al., 2006; Balazadeh et al., 2008b). This observation supports the model that ORS1 is a novel senescence-regulatory transcription factor, although it may regulate fewer genes during senescence than ORE1/ANAC092 (Balazadeh et al., 2010a).

Unraveling the gene regulatory networks controlled by TFs benefits from the identification of *cis*-regulatory elements to which they can bind. To advance our understanding of the downstream regulon of ORS1, we determined its preferred binding site by *in vitro* selection against random-sequence double-stranded oligonucleotides. The preferred ORS1 binding site, constituted of the two core motifs RCGTR (motif 1) and RYACGCAA (motif 2; R = A or G; Y = C or T), separated by a spacer of 4–5 bp, bears similarity with the core binding motif identified for some other NAC transcription factors. Tran

et al. (2004) determined TCnnnnnnnACACG (n representing any nucleotide) as the minimal, and CACG as the core DNA sequence recognized by the three homologous drought-responsive NAC factors ANAC019, 55, and 72. Olsen et al. (2005) described [TA][TG]nCGT[GA] and T[TAG][GA]CGT[GA][TCA][TAG] as binding sites for ANAC019 and ORE1/ANAC092, respectively; both sequences contain the core binding site CGT[GA]. Xue (2005) reported the 23-bp-long element [AG]G[AT]nnCGT[AG]nnnnn[CT]ACGT[AC]A[CT][CT] as consensus sequence recognized by wheat NAC transcription factor TaNAC69.

As ORS1 and ORE1 share 94% sequence identity within their DNA-binding NAM domain, it is possible that both proteins recognize similar *cis*-elements. Indeed, motif 1 of the ORS1 target sequence (RCGTR) is identical to the central part of the known ORE1 binding site (Olsen et al., 2005). However, as the sequence of the proposed motif 2 of the ORE1 binding site is not known at present, a final conclusion with respect to its binding site preference cannot be drawn at this stage.

Onset and progression of senescence are affected by environmental factors such as nitrogen limitation, darkness, excessive light, drought, salinity, or wounding (e.g. Lutts et al., 1996; Buchanan-Wollaston et al., 2005; Munns, 2005; Albacete et al., 2009). In *Arabidopsis*, a significant proportion of the senescence-regulated TFs, namely at least 52 of the 185 genes, is also affected by environmental stress, particularly by salinity (Balazadeh et al., 2008). Several senescence-controlling NAC genes, including *ORE1*, *AtNAP*, and *ORS1*, are also affected by salinity (He et al., 2005; Balazadeh et al., 2008b this report), suggesting that NAC TFs play a prominent role in salt stress-induced plant senescence.

Nitrogen (N) limitation is another factor that triggers early senescence in many plant species (e.g. Smart, 1994; Diaz et al., 2006; Agüera et al., 2010). Recently, Bi et al. (2007) analyzed global gene expression patterns in *Arabidopsis* plants grown for extended periods (3 weeks) under N-sufficient (3 mM nitrate) as well as moderate (1 mM nitrate) or severe (0.3 mM nitrate) N-limiting conditions. Biomass accumulation at moderate N limitation was reduced to ~80% in comparison to 3 mM nitrate condition and chlorophyll content remained almost unaffected (~5% reduction). Under severe N limitation, biomass accumulation was further reduced to ~35% of that of control plants grown under N-sufficient conditions; chlorophyll content was reduced by ~30% of the control level. All three senescence-regulatory NAC TFs, namely *ORS1*, *ORE1*, and *AtNAP*, were found to be up-regulated under severe but not moderate chronic N-limitation stress (see Additional Files 2 and 3 of Bi et al., 2007), indicating that all three NAC genes control senescence not only under optimal (N-sufficient), but also under severe N-limiting conditions.

While all three NAC genes respond to chronic N limitation, differences in response to environmental stresses, abiotic and biotic, exist. Analysis of public data (GENEVESTIGATOR) revealed that *ORE1* is strongly induced by inoculation with conidiospores of the fungal pathogen *Botrytis cinerea* (more than 10-fold after 48 h), or avirulent and virulent strains of the bacterial pathogen *Pseudomonas syringae* (more than 20-fold after 24 h). Notably, however, *ORS1* remained largely unaffected by both pathogenic treatments (less than two-fold induction). Another clear difference is the induction time course of both genes after H_2O_2 treatment. Whereas *ORS1* expression is rapidly induced by H_2O_2 treatment (within 1 h), *ORE1* only responded after 5 h of treatment. These differences in H_2O_2-dependent gene expression were observed at both the transcript level (Balazadeh et al., 2010b) and in promoter–reporter gene fusions (this report), indicating transcriptional control by upstream TF(s).

Although *ORS1*, *ORE1*, and *AtNAP* all regulate senescence, direct upstream TFs controlling their expression have not been reported until today. However, as senescence is a finely tuned process, and modulated by many hormonal and environmental inputs, it is probably fair to assume that multiple TFs, and potentially also global epigenetic programming (Ay et al., 2009), control their expression. For *ORS1*, our promoter deletion analysis demonstrated that a proximal promoter region harbors *cis*-elements relevant for senescence- and H_2O_2-dependent gene expression (Figures 4 and 6). Further studies will be needed to precisely define which of the evolutionary conserved non-coding sequences are indeed functional and which TFs bind to them.

In summary, our findings reported here suggest a concerted evolution of *ORE1* and *ORS1* and underscore the essential role of NAC transcription factors for the control of leaf senescence in Brassicaceae, including *Arabidopsis thaliana*. In the future, it will be important to unravel the yet largely unknown cross-talk of the upstream signaling pathways that control the expression of these transcription factors in the control of plant senescence and the interconnectivity of the downstream gene regulatory networks they govern.

METHODS

General

Standard molecular techniques were performed as described (Sambrook et al., 2001; Skirycz et al., 2006). Oligonucleotide sequences and AGI codes are given in Supplemental Table 4. For computational analyses, the online tools of GENEVESTIGATOR (www.genevestigator.com; Zimmermann et al., 2004), eFP browser (www.bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi; Winter et al., 2007) and the Plant Transcription Factor Database (http://plntfdb.bio.uni-potsdam.de/v3.0/; Pérez-Rodríguez et al., 2010) were used.

Plants

Seeds of *Arabidopsis thaliana* (L.) Heynh. accessions Col-0, Lip-0, and N13 were obtained from the Arabidopsis thaliana Resource Centre for Genomics (INRA, France; http://dbsgap.versailles.inra.fr/publiclines/). The *ors1-1* T-DNA insertion line was obtained from the GABI-Kat collection (id. 778C04). The *ors1-1/anac092-1* double mutant was obtained by crossing

the single-gene mutants. Seedlings were grown in soil (Einheitserde GS90; Gebrüder Patzer, Sinntal-Jossa, Germany) in a climate chamber with an 8-h day length provided by fluorescent light at 100 μmol m^{-2} s^{-1} and a day/night temperature of 20/16°C and a relative humidity (RH) of 60/75%. For growth under long-day conditions, 2-week-old seedlings where then transferred to a growth chamber with a 16-h day (80 or 120 μmol m^{-2} s^{-1}) and a day/night temperature of 22/16°C and 60/75% RH. For growth under short-day conditions, the light period was reduced to 8 h. Growth in hydroponic culture, salinity treatment, and sample preparation were performed as described using stage 1 plants (28 d old) (Balazadeh et al., 2010a).

Constructs

Prom$_{ORS1}$:GUS fusions: genomics fragments of ~1500, 1000, 585, 400, 230, 204, and 163bp upstream of the *ORS1* translation initiation codon were PCR amplified using primers *ORS1*:GUS–fwd and *ORS1*:GUS–rev, inserted into vector pCR2.1–TOPO (Invitrogen, Karlsruhe, Germany) and then fused via *Bam*HI and *Nco*I sites to the *Staphylococcus* sp. β-glucuronidase (*GUS*) reporter gene in vector pCAMBIA1305.1–Hygromycin (CAMBIA, Canberra, Australia). *35S:ORS1*: *ORS1* open reading frame was amplified by PCR from *Arabidopsis* Col-0 leaf cDNA and inserted into pUni/V5-His–TOPO (Invitrogen). The cDNA was cloned via added *Pme*I/*Pac*I sites into a modified pGreen0229-35S plant transformation vector (Skirycz et al., 2006). *ORS1–RNAi*: a ~200-bp-long *ORS1*-specific DNA fragment encompassing the 3′ part of the coding region and part of the 3′-UTR was amplified by PCR using primers ORS1–fwd and ORS1–rev, inserted into pENTR/D–TOPO vector (Invitrogen) and finally cloned into pJAWOHL8–RNAi vector (kindly provided by Imre Somssich, MPI for Plant Breeding, Cologne, Germany) using GATEWAY cloning. *ORS1–DEX*: *ORS1* cDNA, amplified from leaf cDNA with primers IOE–ORS1–fwd and IOE–ORS1–rev, was inserted into pCR2.1–TOPO and then cloned via *Xba*I and *Bam*HI sites into d143 (Pbi-GR) vector (Lloyd et al., 1994). *ORS1–CELD*: *ORS1* cDNA, PCR-amplified from leaf cDNA with primers ORS1–CELD–fwd and ORS1–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 *ORS1–CELD* in-frame fusion construct, pTacORS1LCELD6XHis. All PCR-amplified DNA fragments were checked by sequencing. *Agrobacterium tumefaciens* strains GV3101 (pMP90) and GV2260 were used for *Arabidopsis thaliana* (Col-0) and *Nicotiana tabacum* L. cv. Samsun NN transformations, respectively. The *ORS1–RNAi* construct was transformed into *Arabidopsis* using *Agrobacterium* strain GV3101RK (pMP90).

Expression Profiling by qRT–PCR

Total RNA extraction, cDNA synthesis, and qRT–PCR were performed as described (Caldana et al., 2007; Balazadeh et al., 2008b, 2010a).

DNA Binding Site Selection

Binding site selection was performed using the CELD system (Xue, 2005) with pTacORS1LCELD6XHis construct, employing biotin-labeled double-stranded oligonucleotide pools Bio-RS-Oligo 1 and Bio-RS-Oligo 3 containing 30-nt random sequences (Supplemental Table 4). ORS1-selected oligonucleotides were cloned and sequenced. The DNA-binding activity of ORS1–CELD protein was measured using methylumbelliferyl β-D-cellobioside (MUC) as substrate (Xue, 2002). DNA-binding assays with a biotin-labeled single-stranded oligonucleotide or a biotin-labeled double-stranded oligonucleotide without a target binding site were used as controls.

Microarray Experiments

Nuclear targeting of ORS1 was induced in *ORS1–DEX* plants by spraying 46-day-old plants with 30 μM dexamethasone or control solution (0.5% ethanol) and harvesting after 5 h. For gene expression analyses of the *ors1-1* mutant, plants were grown in soil and fully expanded leaf number 11 was harvested 38 d after sowing (DAS). Expression data were submitted to the NCBI Gene Expression Omnibus (GEO) repository (www.ncbi.nlm.nih.gov/geo/) under accession number GSE22836. Expression data were analyzed as described (Balazadeh et al., 2010a). Groups were assigned according to the similarity of expression patterns and classified into functional categories using PageMan (Usadel et al., 2006).

Phylogenetic Footprinting

Arabidopsis thaliana cv. Col-0, *Arabidopsis arenosa*, *Brassica oleracea* cv. Capitata alba, *Capsella rubella*, and *Raphanus sativus* cv. Nancy were used (seeds kindly provided by Robert Hasterok and Adam Rostanski, University of Silesia, Katowice, Poland, and Heike Küchmeister, University of Potsdam, Germany) to clone the 5′ proximal parts of *ORS1* and *ORE1* orthologs. The 5′ proximal parts of *ORS1* and *ORE1* orthologs from the species indicated were amplified by PCR: 95°C for 2 min; 40 cycles of 95°C for 45 s, 55°C for 30 s, 72°C for 2 min; 72°C for 5 min. The forward primers (ANAC059F, ANAC092F) were complementary to conserved non-coding sequences of *ORS1* and *ORE1* promoters. The reverse primer (ANAC059/092R) annealed to a conserved region downstream of the translational start codon of both genes. PCR products were purified and sequenced. The sequences obtained were trimmed at the primer binding sites and subjected to comparative analyses. Pair-wise alignments of promoter sequences were performed using ConSite (Sandelin et al., 2004), employing a 50-nucleotide sliding window. The significance of the results obtained was tested by generating random sets of unrelated sequences analyzed with ConSite. This allowed establishing threshold levels of promoter conservation values. The mean background level of 'promoter conservation' in the case of random sequences was 48%. All results of promoter conservation in this study were statistically significant and ranged from 70 to 98%. Detailed analysis of motif conservation across all

promoters was performed using FootPrinter (Blanchette and Tompa, 2003). Different parameters of minimal motif sizes (MMS) and maximum number of mutations allowed within the motifs (MNM) were tested, including MMS/MNM parameters of 11/2, 12/2, and 10/0. We used the PLACE database (Higo et al., 1999) to search for known *cis*-acting elements in the fully conserved sequences of *ORS1* and *ORE1* orthologs. Prior to promoter annotation, all elements ⩽4 bp were filtered out. GenBank accession numbers of promoter sequences are given in Supplemental Table 4.

Other Methods

Histochemical GUS assays was performed as described (Plesch et al., 2001). Fluorometric GUS assays were performed using 4-methyl umbelliferyl β-D-glucuronide (4-MUG; Sigma-Aldrich, Deisenhofen, Germany) as substrate (Jefferson, 1987). Chlorophyll content and ion leakage were determined as described (Balazadeh et al., 2010a). For H_2O_2 treatment, 2-week-old seedlings were incubated for 1 or 5 h in liquid MS medium containing 10 mM H_2O_2.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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REFERENCES

Agüera, E., Cabello, P., and de la Haba, P. (2010). Induction of leaf senescence by low nitrogen nutrition in sunflower (*Helianthus annuus*) plants. Physiol Plant. **138,** 256–267.

Albacete, A., Martínez-Andújar, C., Ghanem, M.E., Acosta, M., Sánchez-Bravo, J., Asins, M.J., Cuartero, J., Lutts, S., Dodd, I.C., and Pérez-Alfocea, F. (2009). Rootstock-mediated changes in xylem ionic and hormonal status are correlated with delayed leaf senescence, and increased leaf area and crop productivity in salinized tomato. Plant Cell Environ. **32,** 928–938.

Andersson, A., et al. (2004). A transcriptional timetable of autumn senescence. Genome Biol. **5,** R24.

Ay, N., Irmler, K., Fischer, A., Uhlemann, R., Reuter, G., and Humbeck, K. (2009). Epigenetic programming via histone methylation at WRKY53 controls leaf senescence in *Arabidopsis thaliana*. Plant J. **58,** 333–346.

Balazadeh, S., Parlitz, S., Mueller-Roeber, B., and Meyer, R.C. (2008a). Natural variation for developmental leaf and plant senescence in *Arabidopsis thaliana*. Plant Biol. **Suppl 1**, 136–147.

Balazadeh, S., Riaño-Pachón, D.M., and Mueller-Roeber, B. (2008b). Transcription factors regulating leaf senescence in *Arabidopsis thaliana*. Plant Biol. **Suppl 1**, 63–75.

Balazadeh, S., Siddiqui, H., Allu, A.D., Matallana-Ramirez, L.P., Caldana, C., Mehrnia, M., Zanor, M.-I., Köhler, B., and Mueller-Roeber, B. (2010a). Gene regulatory network controlled by NAC transcription factor ANAC092/AtNAC2/ORE1 during salt-promoted senescence. Plant J. **62,** 250–264.

Balazadeh, S., Wu, A., and Mueller-Roeber, B. (2010b). Salt-triggered expression of the ANAC092-dependent senescence regulon in *Arabidopsis thaliana*. Plant Sig. & Behav. **5 (6)**.

Bi, Y.-M., Wang, R.-L., Zhu, T., and Rothstein, S.J. (2007). Global transcription profiling reveals differential responses to chronic nitrogen stress and putative nitrogen regulatory components in Arabidopsis. BMC Genomics. **8,** 281.

Blanchette, M., and Tompa, M. (2003). FootPrinter: a program designed for phylogenetic footprinting. Nucl. Acids Res. **31,** 3840–3842.

Bleecker, A.B., and Patterson, S.E. (1997). Last exit: senescence, abscission, and meristem arrest in *Arabidopsis*. Plant Cell. **9,** 1169–1179.

Buchanan-Wollaston, V., et al. (2005). Comparative transcriptome analysis reveals significant differences in gene expression and signalling pathways between developmental and dark/starvation-induced senescence in *Arabidopsis*. Plant J. **42,** 567–585.

Caldana, C., Scheible, W.R., Mueller-Roeber, B., and Ruzicic, S. (2007). A quantitative RT–PCR platform for high-throughput expression profiling of 2500 rice transcription factors. Plant Methods. **3,** 7.

Davletova, S., Schlauch, K., Coutu, J., and Mittler, R. (2005). The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in *Arabidopsis*. Plant Physiol. **139,** 847–856.

Diaz, C., Saliba-Colombani, V., Loudet, O., Belluomo, P., Moreau, L., Daniel-Vedele, F., Morot-Gaudry, J.F., and Masclaux-Daubresse, C. (2006). Leaf yellowing and anthocyanin accumulation are two genetically independent strategies in response to nitrogen limitation in *Arabidopsis thaliana*. Plant Cell Physiol. **47,** 74–83.

Gadjev, I., Vanderauwera, S., Gechev, T.S., Laloi, C., Minkov, I.N., Shulaev, V., Apel, K., Inzé, D., Mittler, R., and Van Breusegem, F. (2006). Transcriptomic footprints disclose specificity of reactive oxygen species signaling in *Arabidopsis*. Plant Physiol. **141,** 436–445.

Gechev, T.S., and Hille, J. (2005). Hydrogen peroxide as a signal controlling plant programmed cell death. J. Cell. Biol. **168,** 17–20.

Gepstein, S., Sabehi, G., Carp, M.J., Hajouj, T., Nesher, M.F., Yariv, I., Dor, C., and Bassani, M. (2003). Large-scale identification of leaf senescence-associated genes. Plant J. **36,** 629–642.

Gregersen, P.L., and Holm, P.B. (2007). Transcriptome analysis of senescence in the flag leaf of wheat (*Triticum aestivum* L.). Plant Biotech. J. **5,** 192–206.

Guo, Y., and Gan, S. (2006). AtNAP, a NAC family transcription factor, has an important role in leaf senescence. Plant J. **46,** 601–612.

Guo, Y., Cai, Z., and Gan, S. (2004). Transcriptome of *Arabidopsis* leaf senescence. Plant Cell Environ. **27,** 521–549.

He, X.J., Mu, R.L., Cao, W.H., Zhang, Z.G., Zhang, J.S., and Chen, S.Y. (2005). AtNAC2, a transcription factor downstream of ethylene and auxin signaling pathways, is involved in salt stress response and lateral root development. Plant J. **44,** 903–916.

Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T. (1999). Plant *cis*-acting regulatory DNA elements (PLACE) database: 1999. Nucl. Acids Res. **27,** 297–300.

Jefferson, R.A. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. Plant Mol. Biol. Rep. **5,** 387–405.

Kim, J.H., Woo, H.R., Kim, J., Lim, P.O., Lee, I.C., Choi, S.H., Hwang, D., and Nam, H.G. (2009). Trifurcate feed-forward regulation of age-dependent cell death involving *miR164* in *Arabidopsis*. Science. **323,** 1053–1057.

Lim, P.O., Kim, H.J., and Nam, H.G. (2007). Leaf senescence. Ann. Rev. Plant Biol. **58,** 115–136.

Lloyd, A.M., Schena, M., Walbot, V., and Davis, R.W. (1994). Epidermal cell fate determination in *Arabidopsis*: patterns defined by a steroid-inducible regulator. Science. **226,** 436–439.

Lutts, S., Kinet, J.M., and Bouharmont, J. (1996). NaCl-induced senescence in leaves of rice (*Oryza sativa* L.) cultivars differing in salinity resistance. Ann. Bot. **78,** 389–398.

Masclaux-Daubresse, C., Purdy, S., Lemaitre, T., Pourtau, N., Taconnat, L., Renou, J.P., and Wingler, A. (2007). Genetic variation suggests interaction between cold acclimation and metabolic regulation of leaf senescence. Plant Physiol. **143,** 434–446.

Miller, G., Suzuki, N., Ciftci-Yilmaz, S., and Mittler, R. (2009). Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ. **33,** 453–467.

Munns, R. (2005). Genes and salt tolerance: bringing them together. New Phytol. **167,** 645–663.

Olsen, A.N., Ernst, H.A., Leggio, L.L., and Skriver, K. (2005). DNA-binding specificity and molecular functions of NAC transcription factors. Plant Sci. **169,** 785–797.

Ooka, H., et al. (2003). Comprehensive analysis of NAC family genes in *Oryza sativa* and *Arabidopsis thaliana*. DNA Res. **10,** 239–247.

Pérez-Rodríguez, P., Riaño-Pachón, D.M., Guedes Corrêa, L.G., Rensing, S.A., Kersten, B., and Mueller-Roeber, B. (2010). PlnTFDB: updated content and new features of the plant transcription factor database. Nucl. Acids Res. **38 (Database Issue),** D822–D827.

Plesch, G., Ehrhardt, T., and Mueller-Roeber, B. (2001). Involvement of TAAAG elements suggests a role of Dof transcription factors in guard cell-specific gene expression. Plant J. **28,** 455–464.

Pourtau, N., Mares, M., Purdy, S., Quentin, N., Ruel, A., and Wingler, A. (2004). Interactions of abscisic acid and sugar signalling in the regulation of leaf senescence. Planta. **219,** 765–772.

Sambrook, J., Fritsche, E.F., and Maniatis, T. (2001). Molecular Cloning: A Laboratory Manual, 3rd edn (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Sandelin, A., Wasserman, W.W., and Lenhard, B. (2004). ConSite: web-based prediction of regulatory elements using cross-species comparison. Nucl. Acids Res. **32,** W249–W252.

Skirycz, A., et al. (2006). DOF transcription factor AtDof1.1 (OBP2) is part of a regulatory network controlling glucosinolate biosynthesis in *Arabidopsis*. Plant J. **47,** 10–24.

Smart, C.M. (1994). Gene expression during leaf senescence. New Phytol. **126,** 419–448.

Tran, L.S., Nakashima, K., Sakuma, Y., Simpson, S.D., Fujita, Y., Maruyama, K., Fujita, M., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2004). Isolation and functional analysis of *Arabidopsis* stress-inducible NAC transcription factors that bind to a drought-responsive *cis*-element in the early responsive to dehydration stress 1 promoter. Plant Cell. **16,** 2481–2498.

Usadel, B., Bläsing, O.E., Gibon, Y., Retzlaff, K., Höhne, M., Günther, M., and Stitt, M. (2008). Global transcript levels respond to small changes of the carbon status during progressive exhaustion of carbohydrates in *Arabidopsis* rosettes. Plant Physiol. **146,** 1834–1861.

Usadel, B., et al. (2006). PageMan: an interactive ontology tool to generate, display, and annotate overview graphs for profiling experiments. BMC Bioinformatics. **7,** 535.

van der Graaff, E., Schwacke, R., Schneider, A., Desimone, M., Flugge, U.I., and Kunze, R. (2006). Transcription analysis of *Arabidopsis* membrane transporters and hormone pathways during developmental and induced leaf senescence. Plant Physiol. **141,** 776–792.

Weaver, L.M., Gan, S., Quirino, B., and Amasino, R.M. (1998). A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. Plant Mol. Biol. **37,** 455–469.

Winter, D., Vinegar, B., Nahal, H., Ammar, R., Wilson, G.V., and Provart, N.J. (2007). An 'electronic fluorescent pictograph' browser for exploring and analyzing large-scale biological data sets. PLoS One. **2,** e718.

Woo, H.R., Chung, K.M., Park, J.-H., Oh, S.A., Ahn, T., Hong, S.H., Jang, S.K., and Nam, H.G. (2001). ORE9, an F-box protein that regulates leaf senescence in *Arabidopsis*. Plant Cell. **13,** 1779–1790.

Xue, G.P. (2002). Characterisation of the DNA-binding profile of barley HvCBF1 using an enzymatic method for rapid, quantitative and high-throughput analysis of the DNA-binding activity. Nucl. Acids Res. **30,** e77.

Xue, G.P. (2005). A CELD-fusion method for rapid determination of the DNA-binding sequence specificity of novel plant DNA-binding proteins. Plant J. **41,** 638–649.

Yang, Y.W., Lai, K.N., Tai, P.Y., and Li, W.H. (1999). Rates of nucleotide substitution in angiosperm mitochondrial DNA sequences and dates of divergence between Brassica and other angiosperm lineages. J. Mol. Evol. **48,** 597–604.

Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W. (2004). GENEVESTIGATOR: *Arabidopsis* microarray database and analysis toolbox. Plant Physiol. **136,** 2621–2632.