ORESARA15, a PLATZ transcription factor, mediates leaf growth and senescence in Arabidopsis

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Received: 10 October 2017
Accepted: 24 May 2018

New Phytologist (2018)
doi: 10.1111/nph.15291

Key words: Arabidopsis, cell proliferation, G1F1/AN3, GRF/GIF regulatory module, leaf growth, leaf senescence, ORE15, PLATZ.

Summary

- Plant leaves undergo a series of developmental changes from leaf primordium initiation through growth and maturation to senescence throughout their life span. Although the mechanisms underlying leaf senescence have been intensively elucidated, our knowledge of the interrelationship between early leaf development and senescence is still fragmentary.
- We isolated the oresara15-1Dominant (ore15-1D) mutant, which had an extended leaf longevity and an enlarged leaf size, from activation-tagged lines of Arabidopsis. Plasmid rescue identified that ORE15 encodes a PLANT A/T-RICH SEQUENCE- AND ZINC-BINDING PROTEIN family transcription factor. Phenotypes of ore15-1D and ore15-2, a loss-of-function mutant, were evaluated through physiological and anatomical analyses. Microarray, quantitative reverse transcription polymerase chain reaction, and chromatin immunoprecipitation as well as genetic analysis were employed to reveal the molecular mechanism of ORE15 in the regulation of leaf growth and senescence.
- ORE15 enhanced leaf growth by promoting the rate and duration of cell proliferation in the earlier stage and suppressed leaf senescence in the later stage by modulating the GROWTH-REGULATING FACTOR (GRF)/GRF-INTERACTING FACTOR regulatory pathway.
- Our study highlighted a molecular conjunction through ORE15 between growth and senescence, which are two temporally separate developmental processes during leaf life span.

Introduction

Leaf senescence, the last developmental stage of plant leaves, is a genetically controlled process that is critical for better plant fitness and survival as it allows for the efficient relocation of nutrients accumulated during the growth phase to developing seeds or other parts (Masclaux et al., 2000; Watanabe et al., 2013). Leaf senescence involves massive but orderly changes in molecular, physiological, and biochemical events (Woo et al., 2013). The onset of leaf senescence requires a certain developmental age range but is also modulated by various intrinsic and environmental factors (Lim et al., 2007a; Guo, 2013). After onset, senescence in leaves progresses rather slowly, compared with acute developmental or defense-related programmed cell death (Buchanan-Wollaston et al., 2005). Such slow progression is achieved by a gradual shift in the balance between self-maintenance activity and degeneration activity, which interact antagonistically with each other throughout the leaf’s life span (Nam, 1997; Woo et al., 2013).

Throughout their life span, leaves undergo a series of developmental changes that require intercoordinated temporal and spatial regulations (Watanabe et al., 2013; Woo et al., 2016). A leaf starts from a leaf primordium and develops into a photosynthetic organ through vegetative growth and maturation, which is accomplished by a tightly coordinated interaction of cell division, expansion, and differentiation, and eventually enters the senescence stage (Lim et al., 2007a; Gonzalez et al., 2012). In this regard, leaf senescence is better understood as the later stage in a continual process of leaf development, not a separate process from early leaf development, and is influenced by alterations in the early developmental and/or photosynthetic period. Nonetheless, most studies on leaf senescence have mainly focused on the stage from leaf maturation to death (Guo, 2013).

Although considerably less is known about the interconnection between early leaf development and senescence, studies on key components of cytokinin or auxin signaling have demonstrated the relevance between the control of leaf growth and senescence by plant hormones. For example, combinatorial double and triple mutations of Arabidopsis HISTIDINE KINASE 2 (AHK2), AHK3, and AHK4 result in a smaller leaf size, as a result of reduced cell proliferation and early leaf senescence, indicating the roles of these receptors in the control of leaf growth and cytokinin-dependent retardation of senescence (Riefler et al., 2006). In addition, cytokinin response factors (CRFs) that act
downstream of cytokinin perception have been implicated in the control of leaf growth and senescence in Arabidopsis (Raines et al., 2016). Similarly, the mutation in AUXIN RESPONSE FACTOR 2 (ARF2), a transcription repressor for early auxin-responsive genes, enhances leaf growth by promoting cell proliferation activity and retards leaf senescence (Okushima et al., 2005; Lim et al., 2010).

Other lines of evidence on the potential contribution of leaf growth on senescence were provided by studies on growth regulators that are required for early leaf development. It has been reported that GROWTH-REGULATING FACTORS (GRFs) and GRF-INTERACTING FACTORS (GIFs) function as transcriptional regulatory complexes and are required for essential aspects of plant growth and development, including leaf development (Gonzalez & Inze, 2015; Kim & Tsukaya, 2015). Mutations in GRF3 or GRF5 reduce leaf size through decreased cell proliferation activity; they also accelerate leaf senescence (Debernardi et al., 2014; Vercruyssen et al., 2015). In addition, a mutant of the GIF1 gene, also known as ANGUSTIFOLIA3 (AN3), exhibits similar phenotypes shown in the grf3 and grf5 mutants (Horiguchi et al., 2005; Debernardi et al., 2014). AINTEGUMENTA (ANT), another important leaf growth regulator through cell proliferation, has been implicated in the regulation of age-dependent leaf senescence by acting downstream of ARF2 in Arabidopsis (Mizukami & Fischer, 2000; Feng et al., 2016).

Although intensive efforts have been dedicated to revealing the molecular mechanisms underlying early leaf development or senescence during the last few decades, no targeted mutant screening has been reported to identify the molecular genetic components that integrate the control of leaf growth and senescence to date. With the aim of identifying the genetic loci involved in the integration of growth and senescence in leaves, we screened activation-tagged lines of Arabidopsis and identified the oresara15-1Dominant (ore15-1D) mutant that exhibited an enlarged leaf size and an extended leaf longevity under natural and stressed conditions. Based on our results, we propose that ORE15 might function as a coordinator to interconnect leaf growth and senescence in cooperation with the GRF/GIF regulatory network module.

Materials and Methods

Plant materials, growth conditions, and mutant screening

Arabidopsis thaliana (L.) Heynh. plants were grown in an environmentally controlled growth room (Korea Instruments, Korea) at 22°C with a 16 : 8 h, light : dark photoperiod. Mutants with an extended leaf longevity and enlarged organ size phenotypes were initially screened by visual evaluations in the T1 generation from activation-tagged lines of Arabidopsis (Weigel et al., 2000). The ORE15 entry clone was prepared by reverse transcription polymerase chain reaction (RT-PCR) of the 732 bp ORE15 coding sequence and subcloning of the resultant PCR product into the pCR-CCD-F vector (Kim et al., 2013). The pGVMV: ORE15-HA fusion cassette was established by LR recombination (Gateway; Invitrogen) using the ORE15 entry clone and gateway version of pGVMV-HA3-N-1300. The ore15-2 (SALK_029507) and ore15-3 (SALK_114243) mutants were obtained from the Salk collection (Alonso et al., 2003). The an3-4 mutant has been described previously (Horiguchi et al., 2005). Double mutants were generated by genetic crossing of ore15-2 with an3-4 and confirmed by PCR-based genotyping (Supporting Information Table S1).

Leaf senescence assays under natural and stress conditions

All senescence experiments were carried out using the third and fourth rosette leaves. Age-dependent leaf senescence was evaluated as described previously (Woo et al., 2001). For the salt- or dark-induced leaf senescence assay, leaves at 12 d after emergence (DAE) were detached and floated on a 3 mM MES buffer (pH 5.7) in the presence of 75 or 150 mM NaCl at 22°C with a 16 : 8 h, light : dark photoperiod or in the dark (Woo et al., 2004). The Fv/Fm ratio (Oh et al., 1997) and the electron transport rate (ETR) (Vercruyssen et al., 2015) were determined using a Walz IMAGING-PAM. Membrane ion leakage was determined by measuring the electrolytes that leaked from the leaves as described previously (Woo et al., 2001).

RNA isolation and analysis of gene expression

Total RNA was extracted using WelPrep (Welgene, Gyeongsan, Korea) and cDNA was synthesized using the ImProm II™ system (Promega) following the manufacturer’s instructions. Quantitative RT-PCR (qRT-PCR) analysis was performed to determine gene expression levels (CFX96 system; Bio-Rad). Transcript abundances of target genes were analyzed using the comparative threshold method, with ACTIN2 (ACT2; AAt3g18780) as the internal control (Kim et al., 2009). Gene-specific primer sets used for qRT-PCR are listed in Table S1.

Microarray experiments

Total RNA was extracted from 4-d-old third and fourth leaves with two biological replicates. Microarray experiments were performed using cDNA amplified from total RNA and the array (SurePrint G3 Arabidopsis GE 4x44K Microarray, G2519F-021169). The log2 intensities of the probes were quantile normalized, and applicable cutoffs for reliable signals in the probes were determined by Gaussian curve fitting (Hwang et al., 2005). Microarray datasets are available in the GEO database (GSE97005).

Gene ontology analysis

The expression level for each gene was calculated by the total sum of expression levels in all transcript variants. Differences in the geometric mean of gene expression level from two biological repeats between (Columbia-0 (Col)) and ore15-2 and between Col and ore15-1D were imported into the MapMan software as data input (log2(fold change values)) with The Arabidopsis
Information Resource (TAIR)10 mapping (Thimm et al., 2004). All genes with reliable expression signals were used for analysis (Table S2). A gene ontology analysis was performed using the PAGE MAN module of the MapMan software package (Usadel et al., 2006). Wilcoxon rank sum tests with a Benjamini–Hochberg correction for multiple tests ($P < 0.05$) were applied for the statistical analysis of gene ontology. In this test, the median fold change of all genes in a particular gene ontology was compared with the median fold change of all other ontologies.

Subcellular localization of ORE15

The pGVMV:ORE15-eGFP fusion cassette was established by LR recombination using the ORE15 entry clone and gateway version of pGVMV-eGFP-N-1300. The resultant plasmid was introduced into Nicotiana benthamiana by Agrobacterium tumefaciens AGL1-mediated infiltration. This tissue was stained with 5 μg ml$^{-1}$ 4',6-diamidino-2-phenylindole (DAPI) for 30 min. The subcellular localization of ORE15-eGFP proteins was observed 2 d after infiltration using Zeiss LSM 510 Meta confocal microscopy.

Anatomy of leaves

For kinematic analysis of leaf growth, Arabidopsis plants were grown on MS medium at 23°C with a 16 : 8 h, light : dark cycle. Kinematic analysis of cell division and expansion in leaves was performed as described previously with minor modifications (Horiguchi et al., 2005; Jun et al., 2013). Leaf area with the maximum width from midvein to leaf margin was photographed under bright field illumination using Axioskop 2 microscope (Zeiss) to obtain paradermal images of the leaf cells. The number of cells in a leaf was determined by counting the cells from the mid-vein to the leaf margin in the maximum width region and then multiplying the leaf area by the number of cells (Fig. S1). Size of leaf cells was determined by measurement of cell area in the central region between midvein and leaf margin (Fig. S1) and 350–2000 cells were used for measurement of cell area in the leaves of individual plants. Relative cell proliferation rate was determined by the first derivative of a quadratic function locally fitted through logarithmic values of cell numbers at three neighboring time points (Kumar et al., 2015).

Chromatin immunoprecipitation (ChIP)-qPCR

A quantity of 2 g of 7-d-old seedlings were crosslinked and their chromatin was extracted, as described in a study by Kim et al. (2014). Immunoprecipitation was performed using anti-HA antibody (Abcam 9110) conjugated with protein A/G magnetic beads and eluted DNA solution was used for qPCR analysis to examine the enrichment of target genes. Primers used are listed in Table S1. Enrichment was calculated by ChiPed samples against input DNA samples. Relative fold-enrichment was calculated by normalizing the individual fold-enrichment of amplicons to that of fold enrichment of Col within each trial.

Materials and methods related to supplementary figures and tables are available in Methods S1.

Results

ore15-1D mutant exhibits increased leaf longevity along with an enlarged organ size

To identify novel genetic regulators that mediate leaf longevity and growth, we screened c. 10 000 T1 activation-tagged lines of Arabidopsis. A dominant mutant showing extended leaf longevity and an enlarged organ size phenotype was isolated and designated as the ore15-1D mutant (Table S3). As shown in Fig. 1(a), the ore15-1D mutant leaves exhibited delayed leaf yellowing compared with wild-type (Col) leaves. To examine detailed leaf senescence phenotypes of ore15-1D, we measured well-established physiological parameters of leaf senescence, including the $F_{v}/F_{m}$ ratio reflecting the photochemical efficiency of photosystem II and ETR in the third and fourth rosette leaves throughout their life span. Col leaves lost 33.2% of their $F_{v}/F_{m}$ ratio at 32 DAE relative to $F_{v}/F_{m}$ ratio at 12 DAE, while a significant reduction of $F_{v}/F_{m}$ ratio in ore15-1D mutant leaves was not evident until 36 DAE (Fig. 1b). Similarly, ETR of Col leaves rapidly declined to 18.9% at 28 DAE, while that of ore15-1D was maintained over 53.9% until 36 DAE (Fig. 1c). ore15-1D mutant leaves also exhibited a slower increase in membrane ion leakage compared with Col leaves, as shown in Fig. 1(d). We further characterized delayed leaf senescence phenotypes in ore15-1D by monitoring the expression of several senescence marker genes. ore15-1D leaves had a lower accumulation of ORESARA1 (ORE1), SENESCENCE4 (SEN4), and SENESCENCE-ASSOCIATED GENE 12 (SAG12) transcripts along the leaf life span compared with Col leaves (Fig. 1e–g).

In addition to increased leaf longevity, all rosette leaves of 3- and 8-wk-old ore15-1D plants were distinctively larger than those of Col plants, although the ore15-1D mutant produced fewer rosette leaves (Fig. 2a). The ore15-1D mutant plants also had an increase of 28.8% in whole-plant height and 15.6% in primary rosette leaves (Fig. 2b). Moreover, seed volume and weight as well as total seed yield per plant of ore15-1D mutant plants were substantially increased (Fig. 2d–f). Collectively, these data indicate that the ore15-1D mutation confers enhanced leaf longevity along with increased organ growth phenotypes throughout plant development.

ore15-1D leaves show delayed stress-induced leaf senescence

Senescence in leaves is primarily governed by age, but can also be modulated by diverse exogenous factors such as pathogen infection, darkness, and environmental stress (Guo & Gan, 2012; Schippers et al., 2015). To investigate the function of ORE15 in stress-induced leaf senescence processes, we examined dark, salt, and oxidative stress-induced senescence phenotypes in the detached leaves of Col and ore15-1D plants. Detached leaves from Col and ore15-1D plants were incubated...
in MES buffer in the darkness, or with or without 150 mM NaCl, or 15 mM H$_2$O$_2$ in the light/dark cycles up to 6 d after treatment (DAT).

Consistent with the delayed senescence phenotypes shown in developmental senescence, decreases in $F_{v}/F_{m}$ ratio and ETR of ore15-1D leaves under dark, salt, and oxidative stress-induced senescence were significantly delayed compared with those in Col leaves (Fig. S2). Taken together, these data suggest that ORE15 plays an important role in regulating leaf senescence triggered by diverse senescence-inducing factors.

ORE15 encodes a PLANT A/T-RICH SEQUENCE- AND ZINC-BINDING PROTEIN (PLATZ) transcription factor

The delayed senescence phenotype of the ore15-1D mutant was tightly cosegregated with a single locus of T-DNA insertion, as the linked phosphinothricin-resistant gene segregated 3 : 1 in the $T_2$ generation (Table S3). Plasmid rescue was carried out to determine the location of T-DNA insertion in the ore15-1D mutant. A sequence analysis of the rescued plasmids revealed that T-DNA resided in the intergenic region on chromosome 1 between At1g31030 and At1g31040 (Fig. 3a). A qRT-PCR analysis showed that the transcript abundance of At1g31040 was elevated 210-fold in ore15-1D, whereas that of At1g31030 remained unaffected (Fig. 3b).

To discern whether the overexpression of At1g31040 causes the mutant phenotypes in ore15-1D, we generated transgenic lines carrying the At1g31040 protein-coding sequence under the control of the cassava vein mosaic virus (CaVMV) promoter in the Col background. As indicated in Fig. S3, transgenic lines overexpressing At1g31040 exhibited an enlarged rosette leaf size as well as delayed senescence phenotype during dark-induced leaf senescence; thus, At1g31040 was designated as the ORE15 gene.
ORE15 encodes a 243-amino-acid protein containing two zinc finger motifs and two nuclear localization signals (NLSs; Fig. 3c) and belongs to the PLANT A/T-RICH SEQUENCE- AND ZINC-BINDING PROTEIN (PLATZ) transcription factor family. The Arabidopsis genome contains 11 genes that encode proteins with a PLATZ domain with 32–47% identity and 45–60% similarity at the amino acid level. A phylogenetic analysis has shown that At2g12646 is the most closely related protein to ORE15 among the PLATZ proteins in Arabidopsis, with a 43.4% sequence identity in an entire protein region (Fig. 3d). As ORE15 contains two putative NLSs (Fig. 3c), we examined whether ORE15 localizes in the nucleus. The ORE15 protein fused with enhanced green fluorescent protein (eGFP) under the control of the CsVMV promoter was transiently expressed in N. benthamiana leaves. The GFP signal was detected in the nucleus that was marked with DAPI staining (Fig. 3e), indicating that ORE15 functions in the nucleus.

The results supporting the fact that ORE15 is involved in leaf senescence programs prompted us to examine expression patterns of ORE15 along leaf age. The ORE15 transcript abundance was the highest in young leaves (at 4 DAE), and was maintained at relatively lower levels as the leaf aged (Fig. 3f). We further examined the expression of ORE15 in various Arabidopsis organs to investigate the possible involvement of ORE15 in other developmental processes. As shown in Fig. 3(g), ORE15 had high expression in seedlings, roots, and flowers, whereas it had a relatively low expression in mature rosettes and cauline leaves. The ORE15 expression pattern implies that ORE15 is associated with a broad range of development programs, including early organ growth and senescence.

The observation of delayed leaf senescence and enlarged leaf size in ore15-1D and ORE15 overexpressors led us to further examine senescence and growth phenotypes associated with loss-of-function mutations. We isolated two T-DNA-insertional mutant lines, ore15-2 and ore15-3, in which T-DNA had been inserted in the first and third introns of the ORE15 gene, respectively and ORE15 transcript abundances were barely detected (Figs 4a, S4). As shown in Fig. 4(b), ore15-2 and ore15-3 had smaller leaves than Col, which is in agreement with the enlarged organ in ore15-1D. We determined whether the two ore15 mutations could alter leaf senescence induced by salt or dark treatment. When we measured the $F_{v}/F_{m}$ ratio and ETR upon salt treatment, the ore15-2 and ore15-3 leaves exhibited earlier senescence phenotype than those of Col (Fig. 4c,d). By contrast, ore15-2 and ore15-3 did not result in significant changes in dark-induced leaf senescence (Fig. 4e,f). Note that, in subsequent
experiments, ore15-2 was used for further analysis as a representative allele of loss-of-function mutants. Together with the observations from ore15-1D and ORE15 overexpressors, this result indicates that ORE15 is a novel negative regulator of leaf senescence processes.

ORE15 promotes leaf growth by enhancing the rate and duration of cell proliferation

As shown in Fig. 1(a), the third and fourth leaves of ore15-1D were distinctively larger than those of Col, and it was also observed that ore15-2 leaves were smaller than Col leaves (Fig. 4b). To further investigate the role of ORE15 in the control of leaf growth, we performed a kinematic analysis of leaf growth in the third rosette leaves of Col, ore15-1D, and ore15-2 plants at 2 d intervals from 2 to 14 DAE, namely from the initiation of leaf primordium to the maturation stage. The ore15-1D leaves were slightly smaller than Col during the initial growth period (2–8 DAE), but were dramatically enlarged after 10 DAE; the size of the ore15-1D leaves reached 121.1% and 150.8% at 12 and 14 DAE, respectively, compared with that of Col leaves (Fig. 5a). By contrast, ore15-2 leaves were substantially smaller
than Col leaves throughout the life span (Fig. 5a). In addition, leaf expansion of ore15-1D was prolonged until 14 DAE, whereas that of the ore15-2 mutant ceased at 10 DAE, 2 d before that of Col plants was completed (Fig. 5a). These results indicate that the leaf growth of ore15-2 was prematurely terminated, whereas the ore15-1D leaf had a prolonged growth period.

Leaf size is determined by an increase in cell number and cell size (Vanhaeren et al., 2015); thus, we measured the number and size of leaf cells to assess the contribution of cell proliferation and expansion to the altered leaf size of the ore15 mutants. The number of palisade cells in ore15-1D started to increase remarkably from 8 DAE, by showing differences from that in Col, and resulted in 2.34% and 2.50% of that in Col leaves at 10 and 14 DAE, respectively (Fig. 5b; Table S4). This corresponded to a higher rate and prolonged duration of cell proliferation in ore15-1D compared with those in Col (Fig. 5c). By contrast, the number of palisade cells in ore15-2 leaves was less than that of Col leaves throughout the leaf growth period examined (Fig. 5b; Table S4), which corresponded to consistently lower cell proliferation rates (Fig. 5c). On the other hand, the size of palisade cells in ore15-1D and ore15-2 leaves was smaller and larger than that of Col leaves, respectively (Fig. 5d; Table S5). These results indicate that altered leaf growth in ore15-1D and ore15-2 was primarily caused by altered cell proliferation rather than cell expansion.

In summary, our data show that ORE15 promotes leaf growth by enhancing the rate and duration of cell proliferation activity during leaf development.

Increased ORE15 induces expression of the genes involved in cell proliferation

To investigate how ORE15 mediates cell proliferation during leaf development, we performed a microarray analysis that compared transcriptome profiles among 4-d-old third and fourth entire rosette leaves of Col, ore15-1D, and ore15-2 plants. Biological processes influenced by ore15-1D or ore15-2 were identified by the Wilcoxon rank sum test in P AGEMAN using differential expression data of entire genes (P < 0.05; see Methods). As ore15-1D and ore15-2 exhibited opposite phenotypes in leaf growth, we focused on the biological processes that were inversely affected in ore15-1D and ore15-2 (Fig. 6a). In agreement with altered cell proliferation activity in ore15-1D and ore15-2 leaves, transcript abundances of genes involved in ‘cell cycle’ and ‘cell division’ were up-regulated and down-regulated in ore15-1D and ore15-2, respectively. In addition, the transcript abundances of genes involved in both ‘protein synthesis’ and ‘DNA synthesis’ increased and decreased in ore15-1D and ore15-2, respectively, which is expected given that cell proliferation-mediated leaf
growth requires the synthesis of major cellular macromolecules such as DNA and proteins. The pentatricopeptide repeat (PPR)-containing protein gene family was the most noticeable gene family in response to aberrant levels of ORE15. Given that PPR proteins function in plastid and mitochondrial RNA editing (Schmitz-Linneweber & Small, 2008), organellar RNA maturations might be another direct or indirect target pathway of ORE15. Gene ontologies inversely affected in two ore15 mutants included ‘stress.biotic.PR-proteins’, ‘hormone metabolism.jasmonate.synthesis-degradation’, ‘hormone metabolism.ethylene’, ‘secondary metabolism.sulphur-containing.glucosinolates’ and ‘misc.glutathione S transferases’, indicating that ORE15 regulates the expression of the genes involved in biotic and abiotic stress response processes in the early leaf developmental stage.

It is well established that cyclins function as key effector output molecules for cell proliferation in organ growth control (Blomme et al., 2014). Interestingly, the Cyclin family genes were found to be a major gene family inversely affected in both ore15-1D and ore15-2 (P<10^-7 in ore15-1D and P<10^-5 in ore15-2; Fig. 6b; Table S6). Transcript abundances in most of the Cyclin family genes were enhanced in the ore15-1D mutant but were diminished in ore15-2, which corresponds to cell numbers and cell proliferation rates of ore15-1D and ore15-2. This indicates that ORE15 mediates the regulation of cyclin-mediated cell proliferation.

To further gain an insight into the molecular mechanisms of ORE15 underlying leaf growth, we then focused on examining the expression profiles of leaf growth regulators that are involved in the control of primordium initiation, meristemoid division, duration and rate of cell proliferation, and duration and rate of cell expansion (Vanhaeren et al., 2015). The expression of ANT, Cyclin D3;1 (CYCD3;1), GIF1/AN3, GRF5, miRNA396a (miR396a), and TCP4 was inversely affected in ore15-1D and ore15-2, although the expression of most growth regulators was marginally changed by either mutation (Figs 6c, S5; Table S7). Interestingly, all of these genes are primarily involved in leaf growth control by regulating the duration and/or rate of cell proliferation rather than cell expansion. We also noticed that the expression of most GRF and GIF family genes that belong to the miR319–TCP4–miR396–GRF/GIF pathway, one of the major genetic pathways of cell proliferation regulation (Powell & Lenhard, 2012), was inversely affected in the two ore15 mutants (P<10^-5 in ore15-1D and ore15-2; Fig. 6d; Table S8). A qRT-PCR analysis confirmed that transcript abundances of the genes encoding positive regulators of duration and/or rate of cell proliferation (ANT, CYCD3;1, GIF1/AN3, and GRF5) were significantly increased and decreased in ore15-1D and ore15-2, respectively, compared with Col (Fig. 6e). By contrast, transcript abundances of miR396a, a negative regulator of cell proliferation duration, were decreased and increased in ore15-1D and ore15-2, respectively (Fig. 6e).

We further investigated whether ORE15 is involved in the regulation of cell proliferation by examining the efficiency of callus formation from hypocotyl explants of Col, ore15-1D, and ore15-2. When we compared the size of the calluses derived from hypocotyl explants at 35 d after transfer to the callus induction...
medium, those from *ore15-1D* and *ore15-2* were significantly larger and smaller compared with those from Col, respectively (Fig. S6). Overall, these results suggest that ORE15 enhances cell proliferation activity, resulting in increased cell numbers and enlarged organs in plants.

ORE15 regulates leaf growth and senescence through the GRF/GIF-mediated pathway

Given the results of *ore15-1D* and *ore15-2* described earlier, we hypothesized that ORE15 is involved in the regulation of leaf growth and senescence through the GRF/GIF-mediated pathway. The genome-wide transcriptome analysis of *ore15-1D* and *ore15-2* further confirmed this hypothesis. Total RNAs extracted from the third and fourth rosette leaves of Arabidopsis Col, *ore15-1D*, and *ore15-2* at 4 d after emergence (DAE) were used for microarray analysis. Significantly changed gene ontologies were determined by Wilcoxon rank sum tests in which the median value of gene expression in a particular gene ontology was compared with distributions of median values of genes in other gene ontologies. Gene ontologies were taken from the MapMan functional classification. Red and blue boxes indicate gene ontologies that are significantly up- and down-regulated, respectively. Statistical significance is expressed as a Z score. (b) Expression of the *Cyclin* family genes in *ore15-1D* and *ore15-2*. Each cell indicates the expression of a gene belonging to the *Cyclin* family. A heat map shows the log2-transformed values of fold change (FC) levels. (c) Expression of the organ growth regulator genes in *ore15-1D* and *ore15-2*. Genes are shown as dots whose colors indicate different cellular regulatory mechanisms, as described in the symbol legend. The plot was drawn with the log-ratio of the gene expression in *ore15-1D* to Col as the x-axis and that of *ore15-2* to Col as the y-axis. Genes significantly affected in *ore15-1D* and *ore15-2* are shown with their names; the cutoff is 0.5 for the x-axis and 0.3 for the y-axis. (d) Expression of the *GIF* and *GRF* family genes in *ore15-1D* and *ore15-2*. Each cell represents the expression of genes belonging to the *GIF* family (*GIF1* to *GIF3*) and *GRF* family (*GRF1* to *GRF9*) derived from microarray data. A heat map shows log2-transformed values of FC ratios. (e) Expression of cell proliferation regulators in the third and fourth rosette leaves of Col, *ore15-1D*, and *ore15-2* at 4 DAE. The transcript abundance of each gene was analyzed by qRT-PCR, normalized to *ACT2*, and shown as relative values to each level in Col. Values in (e) are means ± SE (*n* = 3).
growth and senescence through the GRF/GIF regulatory module. To investigate expression regulation of ORE15 by this genetic pathway, we examined the expression of ORE15 in an3-4, a mutant of the GIF1 gene, one of the major players in the GRF/GIF module. The level of ORE15 transcripts was not altered in an3-4 (Fig. 7a), indicating that ORE15 might function upstream of GIF1/AN3.

We next performed a double mutant analysis between ore15 mutants and an3-4. First, dark-induced leaf senescence phenotypes of ore15-2 an3-4 and ore15-1D an3-4 were assessed by measuring F/Fₐₙ ratio and ETR. The reduction in physiological markers of leaf senescence (F/Fₐₙ ratio, ETR, and Chl contents) and induction of expression of senescence marker genes (ORE1, SEN4, and SAG12) in ore15-2 an3-4 mutant leaves were more severe than those in ore15-2 and an3-4 mutant leaves (Figs 7b,c, S7). By contrast, dark-induced reductions in F/Fₐₙ ratio and ETR in the ore15-1D an3-4 double mutant were similar to those in an3-4 (Fig. S8a,b). These results, together with the transcriptional regulation of GIF1/AN3 by ORE15 (Fig. 7a), suggest that ORE15 regulates leaf senescence through GIF1/AN3. Note that ore15-2 single mutant leaves did not show any senescence phenotype; by contrast, ore15-2 an3-4 mutant leaves exhibited significantly premature leaf senescence, further supporting a bona fide negative role of ORE15 in regulating leaf senescence. Next, we monitored leaf growth of ore15-2 an3-4 and ore15-1D an3-4 by measuring leaf area and cell number. As previously reported, an3-4 plants had smaller leaves and a reduced cell number compared with Col plants (Fig. 7d,e; Feng et al., 2016). ore15-2 an3-4 plants had a much smaller leaf size and cell number, and ore15-1D an3-4 plants showed intermediate phenotypes compared with the two parental lines (Figs 7d,e, S8c,d), indicating a composite regulatory interaction between ORE15 and GIF1/AN3 in the regulation of leaf growth.

To further reveal a potential regulatory interaction between ORE15 and the GRF/GIF regulatory module at the molecular level, we examined direct association of the ORE15 protein on the promoter of the genes in this pathway. Based on the report that At2g01818, a homologous protein of ORE15, binds to the DNA motif (Fig. 7g). These results indicate that ORE15 is involved in cell proliferation-mediated leaf growth control through the GRF/GIF pathway by the direct regulation of GRF1 and GRF4 expression. Taken together, our results suggest a cooperative action of ORE15 and the GRF/GIF regulatory module in the control of leaf growth and senescence.

Discussion

In this study, we isolated the ore15-1D mutant from an activation-tagged pool of Arabidopsis in an effort to identify a genetic integrator of leaf growth and senescence processes. Based on the delayed senescence phenotypes of the ore15-1D mutant (Figs 1, S2), along with the early senescence phenotypes of ore15-2 (Fig. 4), ORE15 is a negative regulator in controlling leaf senescence. Although many positive regulators of leaf senescence have been isolated and functionally implicated in the molecular mechanisms of leaf senescence, relatively few negative regulators have been uncovered up to now (Li et al., 2014). This is mainly because most attempts to identify negative regulators of leaf senescence have been made to screen populations generated by T-DNA, chemical, or irradiation mutagenesis, which primarily give rise to loss-of-function mutations. In such mutant screens, it is quite difficult to distinguish early senescence symptoms caused by defects in bona fide negative regulators of senescence from senescence-like symptoms caused by loss of essential genes for survival or homeostasis maintenance. Here, we screened delayed leaf senescence mutants in an activation-tagged pool, which is known as a powerful and efficient strategy to identify negative genetic elements in the leaf senescence program (Weigel et al., 2000; Lim et al., 2007).

ORE15 is a PLATZ transcription factor family protein

Our subcellular localization experiment indicated that the ORE15 protein is localized in the nucleus (Fig. 3e), which is consistent with the two previously characterized PLATZ proteins, pea PLATZ1 and GmPLATZ1 (Nagano et al., 2001; So et al., 2015). The pea PLATZ1 protein acts as a transcriptional repressor by nonspecifically binding to A/T-rich sequences (Nagano et al., 2001). By contrast, we suggest that ORE15 acts as a transcriptional activator as it positively regulates the expression of GRF1 and GRF4 through direct binding on their promoters containing cis-acting elements of 5’-GAANNNTCNNGA-3’ with nonA/T rich nucleotide compositions (Fig. 7g). This suggests that each PLATZ protein has an intrinsic transcriptional activator or repressor function. However, the possibility that a PLATZ protein acts as both a transcriptional repressor and a transcriptional activator cannot be ruled out, depending on the target DNA sequence and different interacting partner proteins. Genome-wide identification of the target genes of ORE15 and further characterization of their transcriptional activation and/or repression by ORE15 in Arabidopsis will advance our understanding of the molecular basis of how ORE15 can mediate cell proliferation and leaf senescence along life span.

It was observed that ORE15 was preferentially expressed in young leaves (Fig. 3f). This observation coincided with the procell proliferation activity of ORE15 during leaf development (Fig. 5). We also observed dramatic increases in plant height, primary root length, and seed size in the ore15-1D mutant (Fig. 2), differential cell proliferation activities in the hypocotyl calluses of the two ore15 mutants (Fig. S6), and high accumulation of ORE15 transcripts in seedlings, roots, and floral organs (Fig. 3g).
Moreover, PLATZ1 in pea is preferentially expressed in the active cell division regions, including the root tip and terminal buds (Nagano et al., 2001). Based on these observations, we can speculate that the PLATZ transcription factors play important roles in cell proliferation, not only in leaves but also in other organs involving actively dividing cells.
ORE15 might antagonistically function with TCP4 in the miR396–GRF/GIF module for cell proliferation regulation

Based on our kinematic measurement of leaf growth (Fig. 5), we concluded that the altered leaf size observed in ore15-1D and ore15-2 was mainly caused by changes in the rate and duration of cell proliferation. Cell proliferation-mediated leaf growth is coordinate controlled by complex interactions among genetic regulators with regard to developmental stages and environmental factors (Casadevall et al., 2013; Vanhaeren et al., 2015). Among the complicated cell proliferation regulatory networks, the miR319–TCP4–miR396–GRF/GIF pathway has been well characterized; miR319 negatively regulates TCP4 at the posttranscriptional level (Schommer et al., 2014), TCP4 positively regulates miR396 as well as negatively regulating GRF5, GRF6, and GIF1/AN3 independent of miR396 (Rodriguez et al., 2010). Finally, miR396 targets GRF1–4 and GRF7–9. It is also known that GRF1 and GRF3 negatively regulate the expression of miR396 and other GRFs (Hewezi et al., 2012). In this study, we propose a functional model of ORE15 as an antagonistic partner of TCP4 in the regulation of cell proliferation through the miR396–GRF/GIF module (Fig. S9). We have provided several lines of evidence supporting our model in this study. First, expression of the genes that were activated (miR396a) and repressed (the GRF and GIF family genes) by TCP4 was negatively and positively correlated with ORE15 expression, respectively (Fig. 6c–e). Second, ORE15 directly bound to the promoters of GRF1 and GRF4 (Fig. 7g). Third, the transcript abundance of TCP4 was not affected by either of the ore15 mutations (Fig. 6e). Fourth, the transcript abundance of ORE15 was not altered in the an3-4 mutant (Fig. 7a).

The double mutant analyses of an3-4 and ore15 mutants provided further genetic insights into the mechanisms involving ORE15 and GIF1/AN3. The expression of most GRFs and GIFs, including GIF1/AN3, was affected in the two ore15 mutants, and the ORE15 protein was directly associated with GRF1 and GRF4 promoters (Figs 6d, 7f,g), implying crucial roles of ORE15 in the GRF/GIF network. Nevertheless, leaf growth of ore15-2 an3-4 and ore15-1D an3-4 (Figs 7d,e, S8c,d) indicated composite regulatory interaction between ORE15 and GIF1/AN3. This might be a result of the existence of downstream targets that ORE15 and GIF1/AN3 independently regulate, in addition to common targets to which they act cooperatively. Further genetic studies on ORE15 and the genes involved in the miR319–TCP4–miR396–GRF/GIF pathways will help us to unravel the detailed mechanisms on how ORE15 can regulate cell proliferation during early leaf development. Recent investigations have highlighted that GIF1/AN3 is associated with SWI/SNF chromatin remodeling complexes, which is involved in the epigenetic regulation of several growth regulator genes, including GRFs (Vercruyssen et al., 2014; Nelissen et al., 2015). Therefore, further analysis dealing with a possible involvement of the SWI/SNF–GIF1/AN3 module in the epigenetic control of direct targets of ORE15 needs to be undertaken to develop a better picture of the regulation of cell proliferation in leaf development.

Another interesting feature of ORE15 functions was that the ore15 mutations affected the expression of ANT and CYCD3:1 (Fig. 6c,e). In fact, ANT and CYCD3:1 belong to the AUXIN-REGULATED GENE INVOLVED IN ORGAN SIZE (ARGOS)–ANT–CYCD3 pathway, which is another well-characterized pathway involved in cell proliferation-mediated leaf growth (Powell & Lenhard, 2012). We believe that ORE15 functions in this pathway probably through acting upstream of ANT. In addition to CYCD3:1, our microarray analysis indicated that ORE15 affects the expression of most of the Cyclin family genes (Fig. 6b). It is uncertain whether ORE15 is directly involved in regulating the expression of a subset of the Cyclin family genes to induce cell proliferation or whether altered expression of the Cyclin family genes is a consequence of enhanced or attenuated cell proliferation of the ore15 mutants. Further study is required on the detailed mechanisms involved in the regulation by ORE15 of the expression of ANT and Cyclin family genes. So far, to our knowledge, there is no report on the regulators that link the two major pathways corresponding to cell proliferation-mediated regulatory networks for leaf growth. Thus, further studies to investigate how ORE15 links the ARGOS–ANT–CYCD3 and miR319–TCP4–miR396–GRF/GIF pathways are needed.

How does ORE15 regulate both leaf growth and senescence?

Despite the importance of coordinated regulation among diverse developmental processes throughout the life span, our knowledge of the interrelationship between early leaf development and senescence is very limited. Here, we identified that ORE15 mediates cell proliferation and senescence during leaf development. Thus, we believe that ORE15 might provide us with a good opportunity of investigating the mechanisms involved in mediating early leaf development and senescence.

How does ORE15 regulate both leaf growth and senescence, two seemingly unrelated events occurring with a time lag of several weeks? We propose that ORE15 might utilize the miR319–TCP4–miR396–GRF/GIF pathway as a primary means of regulating both leaf growth and senescence. Our genetic analyses suggested that ORE15 primarily utilizes GIF1/AN3 in the regulation of leaf senescence. First, ore15-2 an3-4 double mutant leaves exhibited stronger early senescence phenotypes than any of the single mutants (Figs 7b,c, S7), suggesting the involvement of ORE15 and GIF1/AN3 in the same genetic pathway. Second, the an3-4 mutation completely masked the delayed leaf senescence phenotypes of ore15-1D (Fig. S8a,b), indicating that GIF1/AN3 functions downstream of ORE15. Note that mutations of genes involved in the miR319–TCP4–miR396–GRF/GIF pathway have been reported to be associated with leaf senescence. In addition to GRF3, GRF5, and GIF1/AN3 mentioned earlier, it is known that miR319 regulates leaf senescence through its targets, the TCP transcription factors, partly by modulating jasmonic acid biosynthesis (Schommer et al., 2008). The jaw-D mutant overexpressing miR319a exhibited delayed leaf senescence, while overexpression of TCP4 led to premature leaf senescence.
Prospects of crop improvement for higher productivity by ORE15

Delayed leaf senescence is an agronomically important trait that can be adopted to extend postharvest storage of leafy vegetables and/or to improve seed quality in cereal crops (Wu et al., 2012). Larger organ size is another valuable agricultural trait to increase the productivity of vegetables, fruits, and cereal crops (Doebely et al., 2006). Our data revealed that ORE15 is an important regulator required for leaf growth and senescence control. Indeed, ectopic expression of ORE15 drastically enhanced postharvest leaf life span in the darkness (Fig. S2a,b) and seed yield (Fig. 2d–f). Thus, considering that ORE15 or its homologous proteins are conserved in most of the plant kingdoms, ORE15 can be translated to crop species for increased agricultural productivity. We believe that the genetic engineering of ORE15 would result in yield and quality improvements in leafy vegetables, cereal crops, and even algae.

In conclusion, our data indicate how ORE15 guides cell proliferation during growth and leaf senescence by antagonistically acting with TCP4 to modulate the miR396–GRF/GIF pathway. Taken together, our study highlights the importance of a molecular conjunction between two temporally separate biological processes at early and final developmental stages by revealing the regulatory functions of ORE15.

Acknowledgements

This research was supported by the Institute for Basic Science (IBS-R013-D1), and mid-carrier Researcher Program (2015R1A2A01005820 and 2017R1A2B4012714) through the National Research Foundation of Korea (NRF) funded by Ministry of Science and ICT (MSIT), and the Woo Jang-Choon Project (PJ01093902) of the Rural Development Administration (RDA).

Author contributions

J.H.K., J.K., G-T.K. and H.R.W. conceived and designed the study, H.G.N. and H.R.W. conceived the research, and J.H.K., J.K., S.E.J., G.T.K. and H.R.W. wrote the manuscript. J.H.K., J.K., S.E.J., S.P., R.T., D.S.K., Y.K., S-J.P. and J.Y.H. performed the research and analyzed data. All authors reviewed the results and approved the final version of the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article:

**Fig. S1** Scheme of a leaf where cell number and size were measured for kinematic analysis of leaf growth.

**Fig. S2** Delayed leaf senescence in *ore15-1D* under stress conditions.

**Fig. S3** Phenotypes of *ORE15*-overexpressing plants.

**Fig. S4** Expression of *ORE15* in Col, *ore15-2*, and *ore15-3* leaves at 12 DAE.

**Fig. S5** Gene ontology annotation of differentially expressed genes in *ore15-1D* and *ore15-2*.

**Fig. S6** Effect of *ORE15* on cell proliferation competence.

**Fig. S7** Leaf senescence phenotypes in *ore15-2 an3-4* double mutant.

**Fig. S8** Leaf senescence and cell proliferation phenotypes in *ore15-1D an3-4* double mutant.

**Fig. S9** A working model of the functions of *ORE15* in leaf growth and senescence.

**Table S1** List of primers used in this study

**Table S2** Genome-wide transcriptome in Col, *ore15-1D*, and *ore15-2* in early leaf development

**Table S3** Co-segregation of T-DNA locus and senescence phenotype of *ore15-1D* in the T2 generation

**Table S4** Estimated total cell numbers in the leaves of Col, *ore15-1D* and *ore15-2*

**Table S5** Cell size in the leaves of Col, *ore15-1D* and *ore15-2*

**Table S6** Gene expression of *cyclin* family in Col, *ore15-1D* and *ore15-2*

**Table S7** Expression of growth regulator genes in *ore15-1D* and *ore15-2*

**Table S8** Expression of *GIFs* and *GRFs* in *ore15-1D* and *ore15-2*

**Methods S1** Supplemental methods.

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