

RESEARCH PAPER

Auxin response factor 2 (ARF2) plays a major role in regulating auxin-mediated leaf longevity

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Abstract

Auxin regulates a variety of physiological and developmental processes in plants. Although auxin acts as a suppressor of leaf senescence, its exact role in this respect has not been clearly defined, aside from circumstantial evidence. It was found here that *ARF2* functions in the auxin-mediated control of *Arabidopsis* leaf longevity, as discovered by screening EMS mutant pools for a delayed leaf senescence phenotype. Two allelic mutations, *ore14-1* and *14-2*, caused a highly significant delay in all senescence parameters examined, including chlorophyll content, the photochemical efficiency of photosystem II, membrane ion leakage, and the expression of senescence-associated genes. A delay of senescence symptoms was also observed under various senescence-accelerating conditions, where detached leaves were treated with darkness, phytohormones, or oxidative stress. These results indicate that the gene defined by these mutations might be a key regulatory genetic component controlling functional leaf senescence. Map-based cloning of *ORE14* revealed that it encodes ARF2, a member of the auxin response factor (ARF) protein family, which modulates early auxin-induced gene expression in plants. The *ore14/arf2* mutation also conferred an increased sensitivity to exogenous auxin in hypocotyl growth inhibition, thereby demonstrating that ARF2 is a repressor of auxin signalling. Therefore, the *ore14/arf2* lesion appears to cause reduced repression of auxin signalling with increased auxin sensitivity, leading to delayed senescence. Altogether, our data suggest that ARF2 positively regulates leaf senescence in *Arabidopsis*.

Key words: Ageing, *Arabidopsis*, ARF2, auxin, leaf longevity, leaf senescence.

Introduction

Senescence is the process of age-dependent programmed cell death that occurs at the cellular, tissue, organ, or organismal level, leading to the end of a lifespan (Noodén, 1988). As with many other biological processes, plant senescence is developmentally programmed and involves orderly, sequential changes in cellular physiology, biochemistry, and gene expression (Bleecker and Patterson, 1997; Nam, 1997; Buchanan-Wollaston *et al.*, 2003; Lim *et al.*, 2007a).

Senescence is the last stage in leaf development, during which cells undergo a major transition from carbon assimilation and other anabolic reactions to a catabolic pattern that results in cell dysfunction, structural disintegration and cell death (Hajouj *et al.*, 2000). The most visible characteristics are colour changes, as in autumn foliage or the yellowing of leaves in monocarpic plants. This is due to a preferential breakdown of chlorophyll during chloroplast degradation and the synthesis of other pigments. Other

metabolic changes include the hydrolysis of macromolecules such as lipids, proteins, and nucleic acids, which accumulated during the growth phase, followed by remobilization to young leaves and reproductive organs. Thus, although it is a deteriorative cellular process, leaf senescence critically contributes to the fitness of whole plants by ensuring their better survival and the optimal production of offspring (Noodén, 1988; Nam, 1997; Lim *et al.*, 2007a).

Because of the rapid decline in photosynthetic capacity in leaves, senescence limits crop yields and plant biomass production. It can also cause post-harvest spoilage, such as leaf yellowing and nutrient loss in vegetable crops. Therefore, studying leaf senescence not only enhances our understanding of a fundamental biological process, but may also provide information on how to control senescence and improve the agricultural traits of crop plants. For example, delayed leaf senescence, with a concomitant preservation of the photosynthetic apparatus in a soybean mutant, increases its seed yield by 44% (Guiamét *et al.*, 1990). Transgenic tobacco with a delayed leaf senescence phenotype also exhibits a 50% improvement in both seed yield and biomass (Gan and Amasino, 1995).

Leaf senescence, as an integral part of plant development, occurs in the last stage of leaf development. It is thus basically governed by developmental age. However, it is also influenced by various internal and environmental signals that are integrated into age information. Environmental factors may include pathogen infection, shading by neighbours, leaf detachment, drought, limited nutrients, extreme temperatures, and oxidative stress. Internal factors may include the formation of reproductive organs and changes in the levels of plant growth regulators (Weaver *et al.*, 1998; Quirino *et al.*, 1999, 2000; Buchanan-Wollaston *et al.*, 2003; Lim *et al.*, 2007a). Recent genomics and genetics studies in *Arabidopsis* have provided many insights into molecular events and their regulation during leaf senescence, thereby revealing the nature of regulatory factors and a highly complex molecular network (Woo *et al.*, 2001; Gepstein *et al.*, 2003; Lin and Wu, 2004; Buchanan-Wollaston *et al.*, 2005; Guo and Gan, 2006; Kim *et al.*, 2006, 2009; van der Graaff *et al.*, 2006; Lim *et al.*, 2007b; Miao *et al.*, 2008; Zhou *et al.*, 2009). In particular, pathways involving the signalling factors sugar and phytohormones, such as cytokinin, ethylene, abscisic acid (ABA), jasmonic acid (JA), salicylic acid, and auxin, are of key importance in senescence (Buchanan-Wollaston *et al.*, 2003, 2005). However, complex interactions among these signalling pathways during senescence have made it difficult to define the actual role and importance of genes within each pathway (He *et al.*, 2001; van der Graaff *et al.*, 2006). Therefore, to date, there is no clear understanding of the key controlling points within senescence signalling networks.

To enhance our knowledge of the molecular basis for leaf senescence, mutagenized *Arabidopsis* populations were screened with ethyl methane sulphonate (EMS) for delayed senescence mutants. This approach allowed us to identify various important positive elements of leaf senescence. Two allelic mutants with markedly delayed leaf senescence were

investigated here. These were designated as *oresara14* (*ore14-1* and *14-2*; *oresara* means ‘long-living’ in Korean). The *ORE14* gene was identified by map-based cloning, and showed that it encodes auxin response factor 2 (ARF2), one of a family of transcription factors that bind to auxin-responsive elements (AuxREs) in the promoters of auxin-regulated genes (Ulmasov *et al.*, 1997; Liscum and Reed, 2002; Guilfoyle and Hagen, 2007). Recent studies have shown that the *arf2* mutation causes a pleiotropic phenotype, including the increased growth of aerial organs and seed size due to extra cell division, the inhibition of floral bud opening, and delays in flowering, leaf senescence, floral organ abscission, and silique ripening (Ellis *et al.*, 2005; Okushima *et al.*, 2005a; Schruff *et al.*, 2006). These results suggest the involvement of ARF2 in regulating several developmental processes including leaf senescence, although the leaf senescence phenotype of the *arf2* mutants was assessed only visually or was minimally analysed with respect to senescence symptoms or markers.

In our study, the in-depth senescence phenotype of the *ore14larf2* mutant was further analysed under various senescence-inducing conditions using many senescence parameters in order to unravel the physiological function of ARF2 in the plant leaf senescence network. Our results showed that *ORE14/ARF2* plays an important role in the senescence process modulated by various senescence accelerating conditions, including leaf detachment in darkness, oxidative stress, and phytohormone treatment (ABA, ethylene, or MJ), as well as in age-dependent senescence. It is also demonstrated that ARF2 is a repressor of auxin signalling. Through the analysis of the *ore14larf2* mutant, it is suggested that the repression of auxin signalling by ARF2 might positively regulate the onset and progression of leaf senescence in *Arabidopsis*. Our results also illustrate that ARF2, among ARF family genes, might be a major player in controlling auxin-mediated leaf longevity.

Materials and methods

Plant materials and isolation of the ore14 mutant

The *ore14* mutants in the *Arabidopsis thaliana* ‘Col’ wild-type background were identified by screening EMS-mutagenized M2 seeds for delayed leaf senescence, as determined by the delayed loss of chlorophyll during the dark-induced senescence of detached leaves (Oh *et al.*, 1997). Isolated mutants were backcrossed twice with wild-type plants and used for all analyses. Plants for physiological experiments were grown in an environmentally controlled growth room (Korea Instrument, Seoul, Korea) with a 16/8 h light/dark cycle at 23 °C.

Assay of age-dependent leaf senescence

Age-dependent leaf senescence was assayed as described by Woo *et al.* (2001). Quantitative measurements were made of physiological and molecular parameters: chlorophyll content, photochemical efficiency, membrane ion leakage, and gene expression. Chlorophyll was extracted from individual leaves by boiling them in 95% ethanol at 80 °C. The concentration per fresh weight of leaf tissue was calculated as described by Lichtenthaler (1987). The photochemical efficiency of Photosystem II (PSII) was deduced from the characteristics of chlorophyll fluorescence (Oh *et al.*, 1997)

using a portable plant efficiency analyser (Hansatech Instruments, Norfolk, England). The ratio of maximum variable fluorescence to maximum fluorescence yield, which corresponds to the potential quantum yield of the PSII photochemical reactions, represented PSII photochemical reactions (Oh *et al.*, 1997). Membrane ion leakage was determined by measuring electrolytes leaked from leaves (Woo *et al.*, 2001). Two leaves were immersed for 3 h in 3 ml of 400 mM mannitol at 23 °C, with gentle shaking, after which initial conductivity was recorded. Total conductivity was determined after boiling for 10 min. Conductivity was expressed as the percentage of initial conductivity versus total conductivity.

Assay of artificially induced leaf senescence

For dark treatments, the third and fourth leaves at 12 d after their emergence (DAE) were detached and floated on 3 mM MES buffer solution (pH 5.7) and incubated at 23 °C in darkness. For hormone treatments, detached leaves were floated in the same buffer in the presence or absence of 50 µM abscisic acid (ABA; Sigma, USA), 100 µM methyl jasmonate (MJ; Sigma, USA), or 50 µM 1-aminocyclopropane-1-carboxylic acid (ACC; Sigma, USA). All hormone treatments were performed at 23 °C under continuous light. For oxidant treatments, detached leaves were floated on MES buffer in the presence or absence of 15 mM hydrogen peroxide. Chlorophyll content and photochemical efficiency were measured as described above.

RNA gel blot analyses

Total RNA was isolated from leaf tissue with Tri-Reagent (Molecular Research Center, USA). Total cellular RNA (10 µg) was size-fractionated by electrophoresis through a 1.2% formaldehyde-agarose gel and transferred onto a nylon membrane. Radiolabelled probes were prepared using a random labelling kit according to the manufacturer's instructions (Amersham, USA). After hybridization, the membranes were washed as previously described (Woo *et al.*, 2001).

Mapping and sequencing

Homozygotes of *ore14-1* in the 'Col' background were crossed with wild-type 'Ler' plants to create a mapping population, and 1391 plants with a delayed leaf senescence phenotype were selected from the F₂ progeny. Genomic DNA from each plant was utilized for mapping the *ORE14* locus relative to the loci of known Cleaved Amplified Polymorphic Sequence (CAPS) markers. New CAPS markers for the fine mapping of that locus were generated in the sequence of the BAC clones MTG10 and K22G18. The region near the mapped *ORE14* locus was PCR-amplified and cloned into the pGEM T-easy vector (Promega, USA). Two independent PCR products were sequenced to identify the mutated sequence.

Inhibition of hypocotyl elongation in response to phytohormones

To measure hypocotyl lengths, *Arabidopsis* seedlings were grown for 4 d in continuous darkness on 0.1× Murashige and Skoog plates that contained various concentrations of NAA (α-naphthaleneacetic acid; Sigma, USA), BA (benzyladenine; Sigma, USA), or ACC (Sigma, USA). The length of hypocotyls was measured from individual plants ($n > 15$), as previously described by Cary *et al.* (1995).

Results

Identification of *Arabidopsis* mutants with delayed leaf senescence

Arabidopsis, a representative monocarpic plant, is a favoured model for molecular and genetic studies of leaf

senescence because its leaves readily undergo easily distinguishable developmental stages and show a well-defined and reproducible senescence programme (Hensel *et al.*, 1993). *Arabidopsis* populations mutagenized with EMS for delayed leaf senescence mutants were screened, which allowed us to identify positive elements of senescence. Initial screening was carried out by visual evaluation of the degree of yellowing caused by chlorophyll loss when detached leaves were incubated in the dark. This technique is widely used for the consistent acceleration of leaf senescence (Oh *et al.*, 1997). One of the mutants, *ore14-1*, which exhibited a delayed leaf senescence phenotype, was selected for further study. This mutation has a pleiotropic effect on vegetative and reproductive development, including the increased growth of aerial organs, inhibited floral bud opening, late flowering, and a delay in stem senescence (Fig. 1). These traits co-segregated with the delayed leaf senescence phenotype (data not shown). Genetic segregation analysis of the senescence phenotype was performed in F₁ and F₂ progeny derived from crosses between wild-type plants and the *ore14-1* mutant. All F₁ plants had normal phenotypes, including senescence symptoms, demonstrating that the *ore14-1* mutation is recessive. In the F₂ generation, 99 plants exhibiting wild-type phenotypes and 29 mutant plants were scored, indicative of 3:1 segregation ($\chi^2=0.37$, $P > 0.05$). This showed that the mutation is inherited as a monogenic recessive trait.

Another mutant, *ore14-2*, with a phenotype similar to that of the *ore14-1* mutant was also isolated. Genetic complementation tests revealed that these two mutations were in fact allelic (data not shown).



Fig. 1. Whole-plant phenotypes of *Arabidopsis* wild-type ('Col', left) and *ore14-1* mutant (right) plants at 35, 46, and 65 d after germination (DAG).

ORE14 is allelic to *ARF2*, which encodes an auxin response factor

The gene responsible for the *ore14-1* mutation was identified by map-based cloning (Fig. 2A). An initial genetic mapping, with CAPS markers, located *ORE14* close to the *LFY* locus on Chromosome 5. CAPS markers were also generated using the genomic sequences of bacterial artificial chromosome clones (Fig. 2A). Among 1391 F_2 progeny, one and two recombinant chromosomes were observed for the markers MTG10-M and K22G18-A, respectively. These mapping data located *ORE14* at approximately 0.03 cM and 0.07 cM from these respective markers (Fig. 2A). The wild-type and mutant nucleotide sequences for the region around the map location of *ORE14* were then compared. A single base change in the *ore14-1* mutant—a G to A substitution—was identified at position 1673 from the translational start of the gene *At5g62000* (Fig. 2B), which encodes auxin response factor 2 (ARF2). This mutation was expected to result in a premature stop codon at position 558 (Trp→stop) in the ARF2 protein. The same region was then sequenced from the *ore14-2* mutant and it was found that a mutation here led to an early termination of translation at position 115 (Arg→stop) (Fig. 2B). Although the levels of *ARF2* transcript of both mutants were comparable to that of wild-type plants (data not shown), these mutations probably cause a complete loss of function through premature trans-

lation termination. We simultaneously investigated a T-DNA insertion line in the *At5g62000* gene, Salk_041472 line (*arf2-5*). The *arf2-5* plants also exhibited phenotypes similar to those with the *ore14* mutation, including delayed leaf senescence (data not shown). Several *arf2* alleles were previously identified and designated as *arf2-1* to *arf2-9* (Ellis et al., 2005; Schruff et al., 2006); the *ore14-1* and *ore14-2* alleles are *arf2-10* and *arf2-11*, respectively.

Auxin response factor 2 (ARF2) belongs to a family of transcription factors that bind to auxin-responsive elements (AuxREs) in the promoters of auxin-regulated genes (Ulmasov et al., 1997). In previous studies utilizing T-DNA insertion lines, the *ARF2* gene was suggested to play a role in several developmental processes, such as cell division, organ growth, flowering, senescence, and abscission (Ellis et al., 2005; Okushima et al., 2005a; Schruff et al., 2006). However, the effect of ARF2 on leaf senescence has not been extensively examined. This led us to examine further the phenotype of the *ore14/arf2* mutant with several senescence parameters under various senescence induction conditions.

The ore14/arf2 mutant exhibits extended leaf longevity during age-dependent senescence

The senescence symptoms of the *ore14-1/arf2-10* mutant were first examined in detail during age-dependent *in*

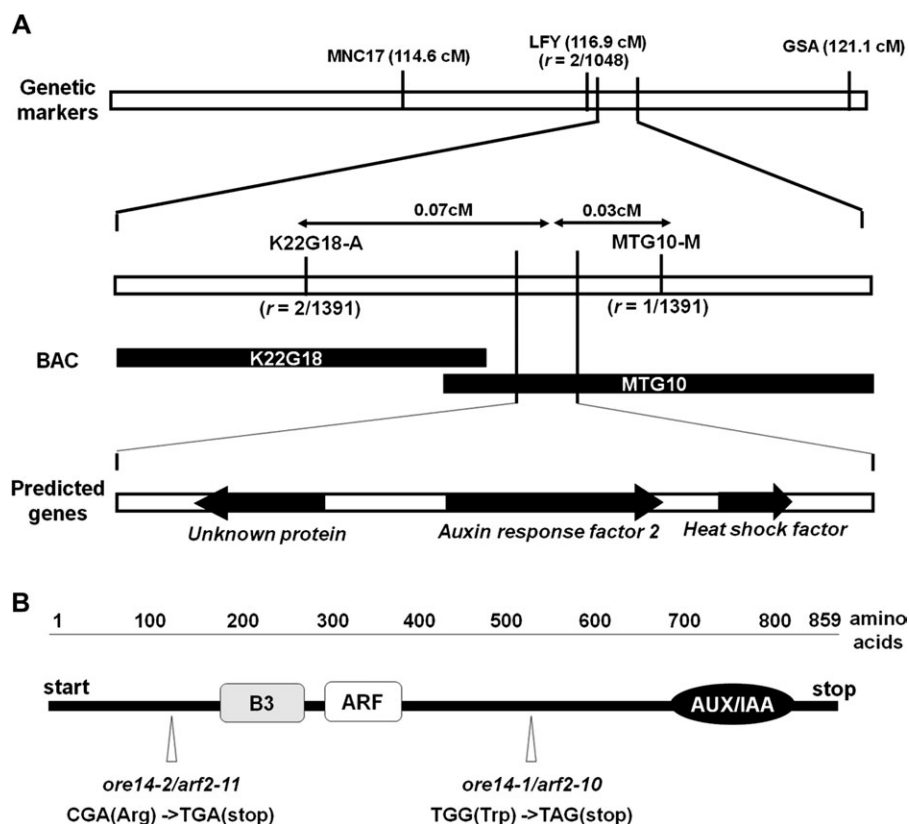


Fig. 2. *ORE14* is auxin response factor 2 (ARF2). (A) Map-based cloning of *ORE14*. The number of recombination events between CAPS markers and the *ORE14* locus (r) is shown; BAC, bacterial artificial chromosome; cM, centimorgan. (B) Schematic representation of *ORE14* with positions of the *ore14-1* and *14-2* mutations; B3, DNA binding domain; ARF, auxin response region; AUX/IAA, domain involved in dimerization with other ARFs or Aux/IAA.

planta senescence. For an accurate assay, two factors were considered. First, in our experiment, leaf senescence was measured on a single leaf base with its age information. Measuring senescence parameters with a mixture of several leaves at a given plant age is not a valid analysis for leaf senescence, since the individual leaves of a plant have different ages. Only the third and fourth rosette leaves of a given plant were used here. The emergence time and growth rate of the fourth leaf was almost identical in wild-type plants and the *ore14-1/arf2-10* mutant. Second, the senescence symptom was measured with various parameters that cover various aspects of senescence physiology.

Leaf yellowing is a convenient visible indicator, mainly reflecting the chloroplast senescence of mesophyll cells. Age-dependent leaf yellowing using the fourth rosette leaf of mutant and wild-type plants were examined first. Wild-type leaves started to turn yellow at the leaf age of 20 d. Leaf age here means the age of the leaf under assay, counting from its visible emergence. After 20 d, yellowing gradually spread to the whole leaf. Wild-type leaves finally showed signs of necrosis at the age of 36 d (Fig. 3A). By contrast, *ore14-1/arf2-10* mutant leaves started to turn yellow at the age of 28 d, i.e. 8 d later than wild-type leaves. They progressed more slowly, so that complete yellowing was not seen in the mutant until 44 d after emergence (DAE). Even then, however, the integrity of the mutant leaf shape was still maintained (Fig. 3A). When the point was determined at which the survival of the entire leaf population per plant was 50%, it was found that the leaf longevity of the *ore14-1/arf2-10* mutant was extended by 35%, from 26 DAE to 35 DAE (Fig. 3B).

Symptoms of age-dependent senescence were then quantitatively investigated by measuring changes in several parameters, such as chlorophyll content, photochemical efficiency, membrane ion leakage, and the expression of senescence-associated genes. Chlorophyll amounts in wild-type leaves started to decrease rapidly after 20 DAE. By the age of 28 d, the chlorophyll content was reduced to 27% of that of 12 DAE leaves (mature green stage). By contrast, losses in the *ore14-1/arf2-10* mutant were slower, with 52% of its chlorophyll remaining at 36 DAE (Fig. 3C). The change in the photochemical efficiency (F_v/F_m) of PSII was also monitored as an index of functional leaf senescence (Oh *et al.*, 1997). As shown in Fig. 3D, the photochemical efficiency of wild-type leaves declined to 38% by the age of 32 d, whereas that of *ore14-1/arf2-10* leaves remained almost unchanged until the age of 32 d and declined only to 39%, even at the age of 44 d. This showed that the functional integrity of PSII was maintained much longer in mutant leaves. Similar observations were made for these two parameters with the *ore14-2/arf2-11* mutant (Fig. 3C, D). Senescence also involves the disruption of plasma membrane integrity as the final step in cell death. This can be conveniently quantified by monitoring ion leakage. It was found that leaf senescence was delayed in the *ore14-1/arf2-10* mutant, as shown by slower increases in membrane ion leakage of the leaves with aging (Fig. 3E).

Moreover, in the *ore14-1/arf2-10* mutant, a photosynthesis-related chlorophyll *alb*-binding protein gene (*CAB*) and a ribulose biphosphate carboxylase small subunit gene (*RBCS*) were expressed at higher levels at the later stages, while the induction of two senescence-associated genes, *SEN4* and *SAG12*, was delayed (Fig. 3F). These results indicated that the *ore14-1/arf2-10* mutation considerably extends leaf longevity and delays leaf senescence, controlling various senescence-associated physiological and molecular symptoms. Thus, these results suggest that ORE14/ARF2 plays an important role in regulating the leaf senescence process.

Delays in artificially induced senescence

Darkness is one of the most potent external stimuli that accelerate leaf senescence. The responses were compared of the *ore14-1/arf2-10* mutant and wild-type plants during dark-induced senescence. To minimize developmental effects, fully grown third and fourth leaves were detached at 12 DAE (mature green stage). Those from the wild-type plants lost 90% of their chlorophyll after 6 d of dark incubation. However, for the mutant, the chlorophyll content declined much more slowly; even after 6 d, over 70% was retained (Fig. 4A, B). Measurement of the photochemical efficiency showed that, after 8 d of incubation, *ore14-1/arf2-10* mutant leaves still maintained over 65% of their initial PSII activity, while wild-type leaves completely lost this activity (Fig. 4C). A relatively slower increase in membrane ion leakage in the *ore14-1/arf2-10* mutant was also observed (Fig. 4D). Consistent with these physiological data, the induction level of *SEN4*, a molecular marker for dark-induced senescence, was much lower in the mutant during dark incubation (Fig. 4E).

It was then tested whether the *ore14-1/arf2-10* mutation affects phytohormone-induced leaf senescence. Three hormones in particular—ABA, MJ, and ethylene—strongly hasten leaf senescence (He *et al.*, 2001; Woo *et al.*, 2001; Lim *et al.*, 2007b). Before examining hormone-induced leaf senescence symptoms, the effect of light alone was evaluated first as a control experiment. When detached leaves were exposed to light in the presence of MES buffer alone, no significant changes in chlorophyll content and PSII activity in wild-type or mutant leaves were observed (Fig. 5A). To determine whether ORE14/ARF2 is involved in the senescence process induced by senescence-accelerating phytohormones, the senescence symptoms of the *ore14-1/arf2-10* mutant were examined after treatment with these hormones. When detached leaves were treated with MJ, ABA, or ethylene, a rapid decrease in chlorophyll was observed in wild-type leaves and, 5 d after incubation, the chlorophyll of wild-type leaves was reduced to less than 20%. However, *ore14-1/arf2-10* mutant leaves retained more than 80% of chlorophyll following treatment with these hormones at 5 d after incubation (Fig. 5B–D). A similar pattern was observed when PSII activity was measured. These data clearly showed a delay of leaf senescence in the *ore14-1/arf2-10* mutant during plant hormone-accelerated

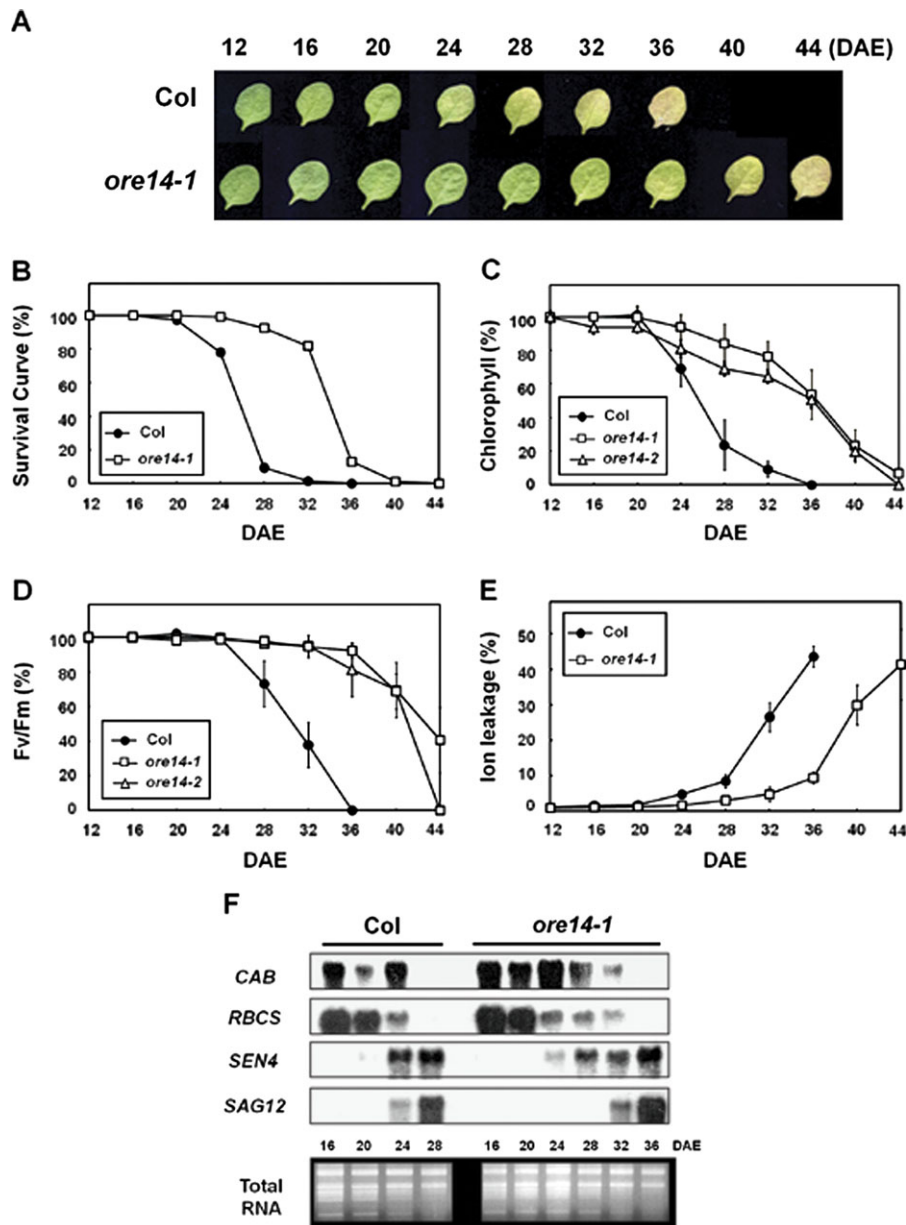


Fig. 3. Extended leaf longevity in *ore14/arf2* mutants. (A) Age-dependent senescence phenotype of the third and fourth rosette leaves of wild-type ('Col') and *ore14-1/arf2-10* mutant plants at different ages. The photographs show representative leaves at each time point. (B) Survival curve. Values indicate the percentage of leaves alive on a given day after emergence (DAE). $n=100$. (C–E) Chlorophyll content (C), photochemical efficiency (F_v/F_m) of PSII (D), and membrane ion leakage (E) were measured from the third and fourth leaves starting at 12 DAE, when leaves had just reached full growth. Error bars indicate standard deviation (SD, $n=30$). (F) Age-dependent changes in gene expression. Total cellular RNAs isolated at the indicated ages were probed with *CAB*, *RBCS*, *SEN4*, and *SAG12*.

senescence. These results imply that ORE14/ARF2 might be a regulator in controlling the senescence process modulated by these hormones, as well as in age-dependent and dark-induced senescence.

Resistance of the *ore14-1/arf2-10* mutant to oxidative stress

Reactive oxygen species play a critical role in mediating oxidative damage and accelerating senescence in a variety of organisms (Reilly *et al.*, 2004). Potential life spans are

positively correlated with plant tolerance to oxidative stress. The oxidative stress tolerance of the *ore14-1/arf2-10* mutant was therefore assessed. For this experiment, the procedure that was used for assaying phytohormone-induced senescence symptoms was adapted and hydrogen peroxide was used as an oxidant. Under such exposure, both chlorophyll content and PSII activity declined rapidly in the wild type. By contrast, the *ore14-1/arf2-10* mutant retained over 80% of its chlorophyll and 95% of the original PSII activity, even at 5 d after treatment (Fig. 6A, B). Thus, the *ore14-1/arf2-10* mutation conferred greater resistance to oxidative stress,

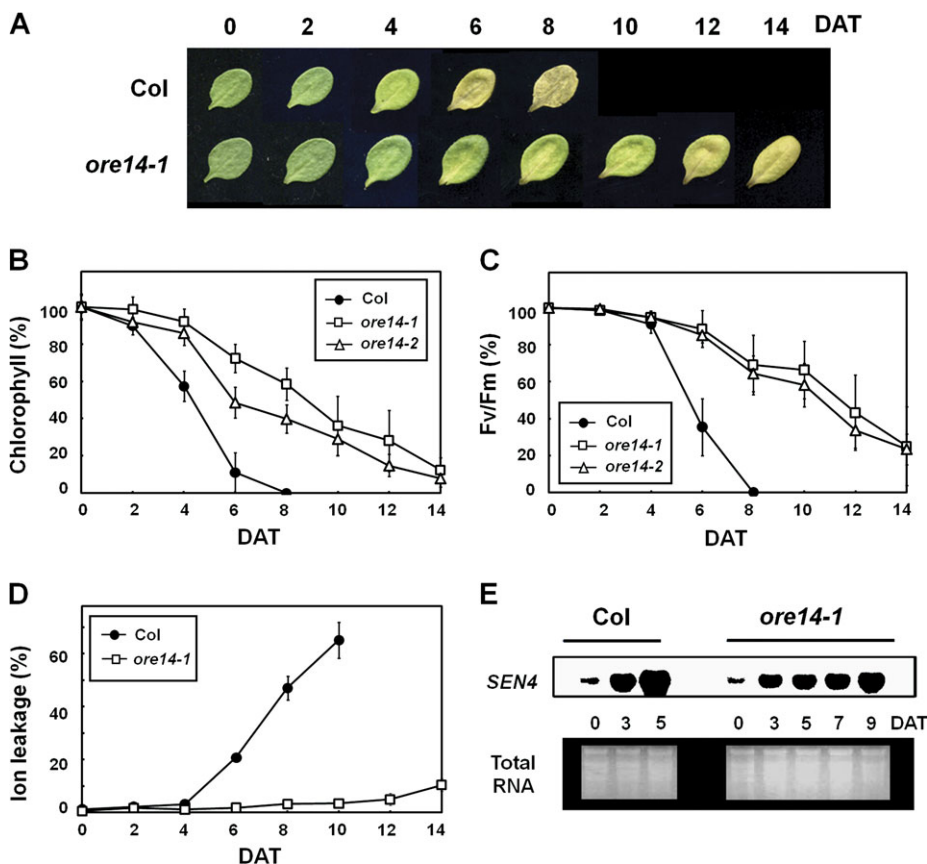


Fig. 4. Delayed leaf senescence of *ore14/arf2* mutants during dark-induced senescence. (A) Phenotypes of wild-type and *ore14* mutant leaves. The third and fourth rosette leaves were detached at the age of 12 d and incubated in darkness; DAT, days after treatment. (B–D) Chlorophyll content (B), photochemical efficiency (F_v/F_m) of PSII (C), and membrane ion leakage (D) were examined every 2 d after dark treatment. Error bars indicate SD ($n=24$). (E) Expression of *SEN4*. Total cellular RNAs were isolated from wild-type and *ore14-1/arf2-10* mutant leaves at the days indicated.

one of the major determinants of senescence. These results further indicate that ORE14/ARF2 might be a central regulator of leaf senescence. The data also support our previous finding that oxidative stress tolerance is linked to the control of leaf longevity (Woo *et al.*, 2004).

ARF2 is a repressor of auxin signalling

The ARF family of transcription factors regulates many responses to auxin. These proteins bind to auxin response elements in the promoters of auxin-regulated genes and activate or repress transcription (Ulmasov *et al.*, 1997). Most ARFs have three domains: an N-terminal DNA-binding domain, a C-terminal dimerization domain, and a middle region (MR) that activates or represses transcription (Ulmasov *et al.*, 1999). ARFs containing glutamine-rich MRs, for example, ARF5 and ARF19, function as activators of auxin-responsive gene expression *in vivo* (Nagpal *et al.*, 2005; Okushima *et al.*, 2005b; Wilmoth *et al.*, 2005) and in transiently transfected protoplasts (Ulmasov *et al.*, 1999). By contrast, ARFs containing proline- and/or serine-rich MRs, such as ARF1 and ARF2, have been suggested to

repress auxin-responsive gene expression in protoplast transient assays (Tiwari *et al.*, 2003, 2004).

To test whether ARF2 represses auxin signalling *in vivo*, the growth responses of *ore14-1/arf2-10* hypocotyls to exogenous phytohormones was examined. In wild-type seedlings, exogenous NAA (a synthetic auxin) inhibits hypocotyl growth. This response was enhanced in the *ore14-1/arf2-10* mutant (Fig. 7A). The hypocotyl growth inhibition responses of the *ore14-1/arf2-10* mutant to cytokinin and ethylene was also examined. Exogenous applications of BA (a synthetic cytokinin) or ACC (an immediate biosynthetic precursor of ethylene) inhibited wild-type hypocotyl growth. However, in contrast to the case with NAA, *ore14-1/arf2-10* hypocotyls showed a nearly normal inhibition response to BA or ACC (Fig. 7B, C), although they were slightly more sensitive at $>1 \mu\text{M}$ ACC. These results indicate that the *ore14-1/arf2-10* mutation does not cause defects in the known signal transduction pathways that underlie the perception and response to ethylene or cytokinin. They also suggest that *ore14-1/arf2-10* preferentially alters the auxin response, at least with regard to hypocotyl growth. Together, these data demonstrate that ARF2 is a true repressor of auxin signalling,

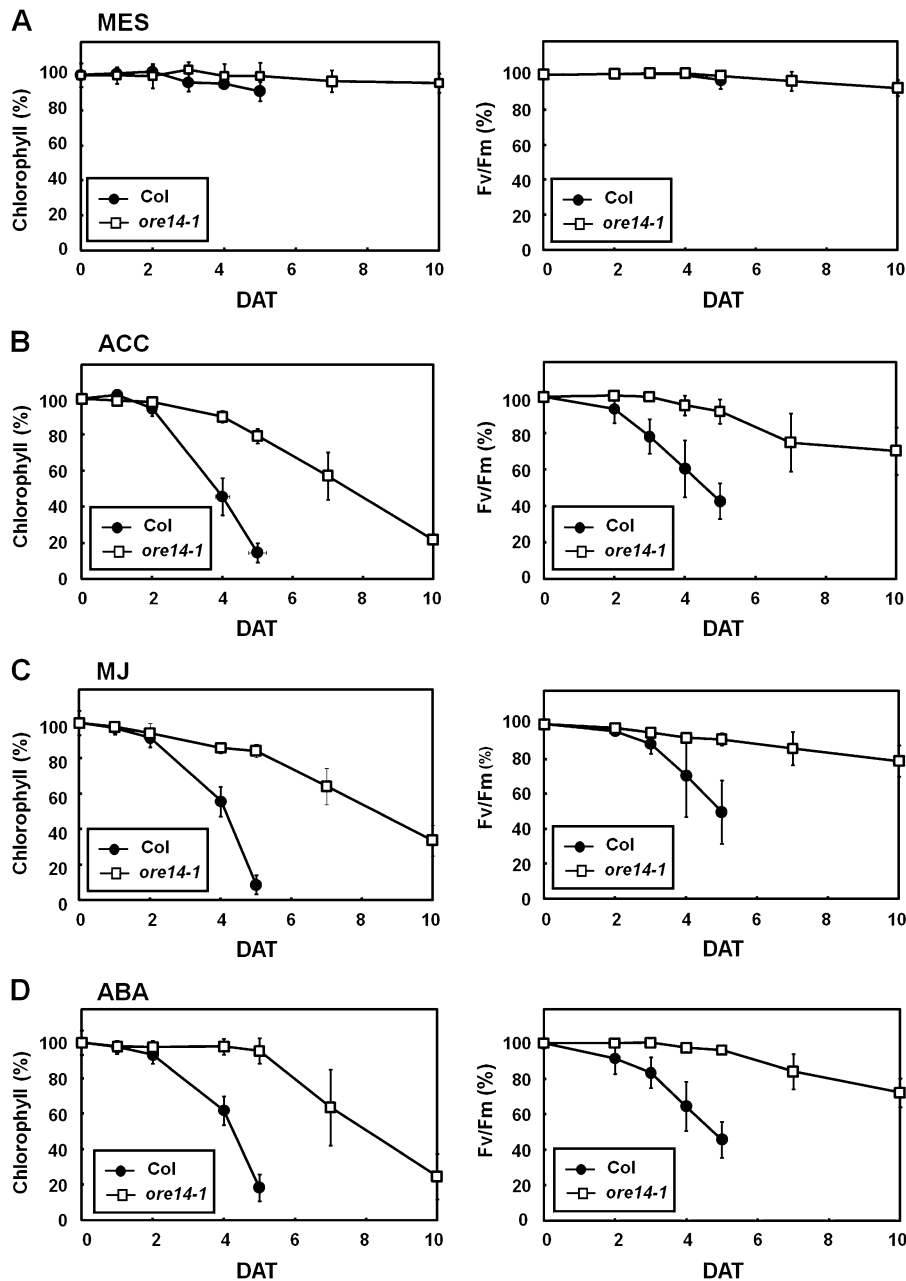


Fig. 5. Delay of leaf senescence in the *ore14-1/arf2-10* mutant during senescence accelerated by phytohormones. The third and fourth rosette leaves were detached at the age of 12 d and incubated under continuous light in MES buffer alone (A), with 50 μ M ACC (B), 100 μ M MJ (C), or 50 μ M ABA (D). Chlorophyll content and photochemical efficiency are presented as average percent values \pm SD, relative to those of leaves incubated in light; $n=24$. DAT, days after treatment.

supporting previous results from protoplast transient assays (Tiwari *et al.*, 2003).

Discussion

ORE14/ARF2 affects a broad spectrum of leaf senescence symptoms

In a screen for *Arabidopsis* mutants exhibiting delayed leaf senescence, two *ore14* mutations were identified, each of which contains a lesion in *ARF2*. These mutations also

confer pleiotropic developmental phenotypes, including delays in flowering and floral organ abscission, and inhibition of floral bud opening in early-formed flowers. These phenotypes have also been reported for other *arf2* mutant alleles (Ellis *et al.*, 2005; Okushima *et al.*, 2005a). The *arf2/mnt1* mutation can also cause the increased growth of aerial organs and more cell divisions (Schruff *et al.*, 2006). The pleiotropic effects of *arf2/mnt1/ore14* mutations may hamper assaying the effect of the gene on senescence. In particular, when minimally analysed with respect to senescence symptoms or markers, for example, the use of a single senescence symptom such as yellowing or loss of

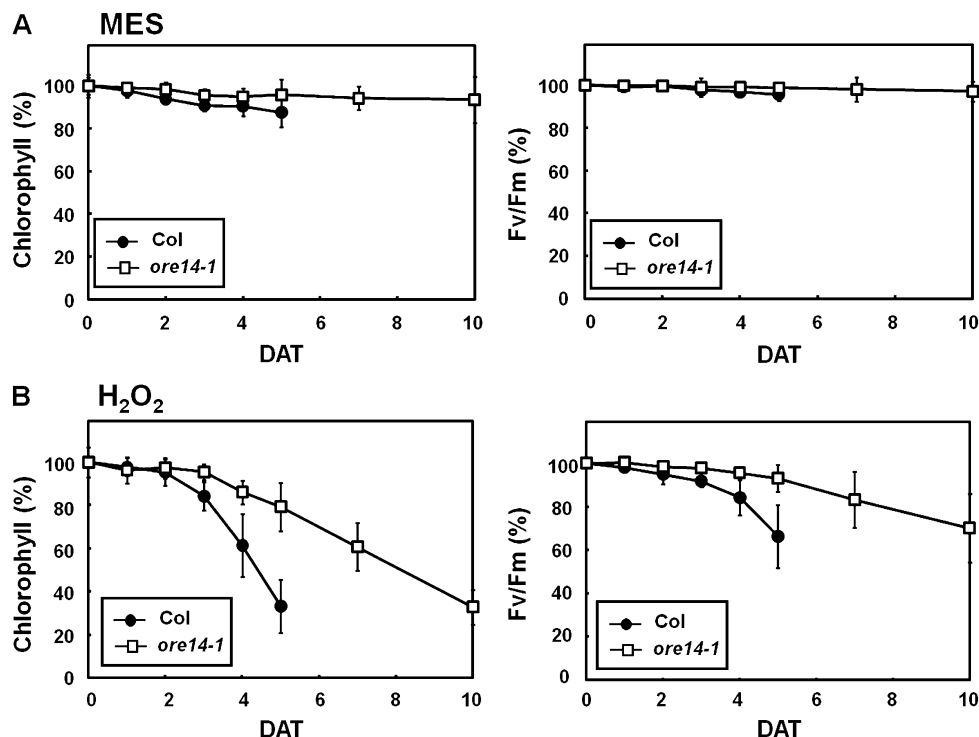


Fig. 6. Delay of leaf senescence in the *ore14-1* mutant during senescence accelerated by oxidative stress. The third and fourth rosette leaves were detached at the age of 12 d and incubated under continuous light in MES buffer alone (A) and with 15 mM hydrogen peroxide (B). Chlorophyll content and photochemical efficiency are presented as average per cent values \pm SD, relative to those of leaves incubated in light ($n=24$). DAT, days after treatment.

chlorophyll, interpretation may be misleading. In order to show that the *ARF2* mutation delays functional senescence, the *ore14* mutations were analysed in detail by examining several anabolic and catabolic parameters. The fact that these mutations influenced all the parameters indicated that the affected plants are functional ‘stay-green’ mutants, and that they are not just delayed in their loss of chlorophyll. This implies that *ORE14/ARF2* might be a key regulatory factor involved in leaf senescence rather than just one of the components that executes the process (Smart, 1994; Oh *et al.*, 1997). It was also found that *ORE14/ARF2* is required for the proper progression of leaf senescence induced by darkness, phytohormones, and oxidative stress, as well as by age. These results suggest that *ORE14/ARF2* functions in a step common to all these processes. The data also support the idea that these senescence-inducing factors, developmental age, dark, hormones, and oxidative stress, all share a common senescence pathway.

The apparently pleiotropic effects of the *arf2* mutation, such as reduced sterility or late flowering, may be associated with delayed leaf senescence, but are unlikely to be its cause. This is because, unlike many other monocarpic species, senescence of individual *Arabidopsis* leaves is not closely linked with the development of reproductive structures (Hensel *et al.*, 1993; Lim *et al.*, 2007a). Both ethylene and cytokinin have a major effect on leaf senescence. Ethylene-insensitive plants, such as *etr1* and *ein2* mutants, have a delayed leaf senescence phenotype (Grbic and Bleeker, 1995). Similarly, *ore12-1*, a gain-of-function mutation in

AHK3 (a cytokinin receptor), confers extended leaf longevity (Kim *et al.*, 2006). To determine whether our *ore14/arf2* phenotype is independent of the ethylene and cytokinin signal transduction pathways, hypocotyl elongation was monitored in response to these hormones. No significant differences were found between mutant and wild-type hypocotyls (Fig. 7), although the mutant exhibited a slightly enhanced sensitivity at high ACC concentrations (1–10 μ M). This enhanced sensitivity might be due to an indirect effect of altered auxin signalling. Nevertheless, it is inferred that the delayed leaf senescence phenotype of *ore14/arf2* plants is not due to alterations in cytokinin or ethylene sensitivity. A similar report was made by Ellis *et al.* (2005), who based their findings on the fact that the *ein2* and *arf2* mutations act additively in delaying leaf senescence symptoms, and that the relative deferment in chlorophyll loss caused by exogenous cytokinin is similar in both wild-type and *arf2* plants. It was also observed that the responses of the *ore14/arf2* seedlings to ABA and MJ were similar to those of wild-type seedlings (data not shown). These results, in turn, indicate that the delayed senescence symptoms of the *ore14/arf2* mutant during phytohormones-induced senescence are not due to defects in the perception of these hormones.

Since leaf senescence is intimately related to previous developmental stages, such as initiation, growth, and maturation, it is possible that genes controlling all these processes, including cell division, could affect age-dependent senescence. This has been demonstrated in leaves from the *bop1-1*

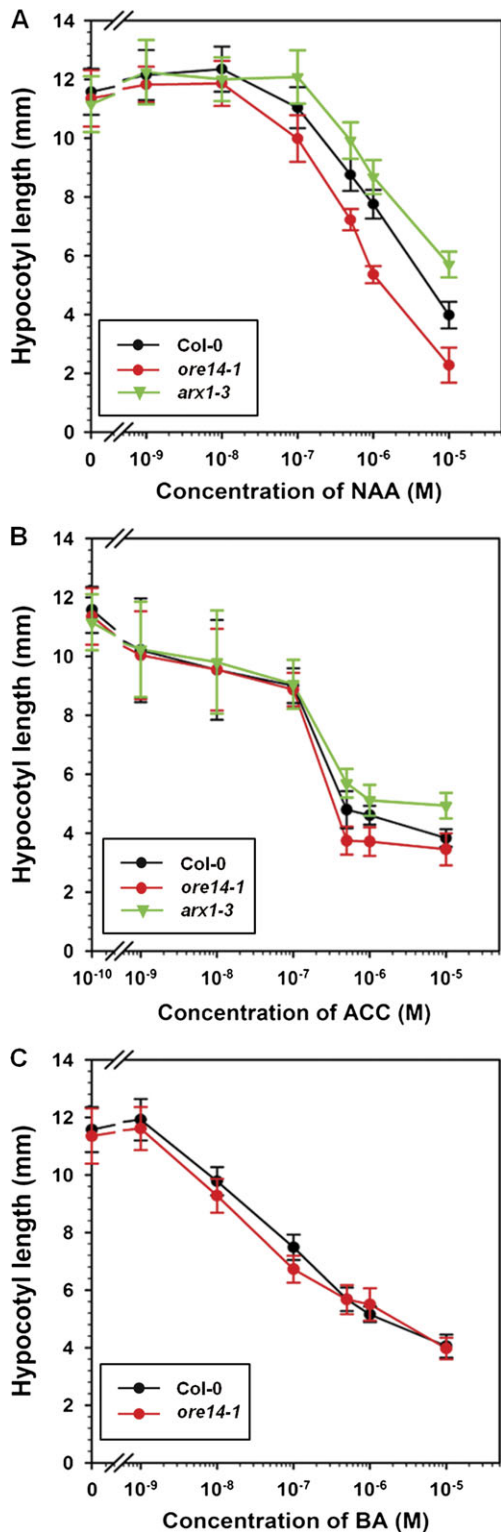


Fig. 7. Hypocotyl growth inhibition responses to exogenous phytohormones. Seedlings of wild type ('Col') and the *ore14-1* mutant were grown for 4 d in various concentrations of NAA (A), ACC (B), or BA (C). The *auxin resistant 1-3* (*axr1-3*) mutant was included as an internal control. At least 20 seedlings were sampled for each treatment. Bars indicate standard errors.

mutant, which has enhanced meristematic activity (Ha *et al.*, 2003). Likewise, organs in *arf2/mnt1/ore14* mutants, including the leaves, are larger than those of wild-type plants, due to extra cell divisions and expansion (Schruff *et al.*, 2006). Thus, it is possible that extended leaf longevity in *ore14* mutants might be related to increased cell division activity. However, because the delayed senescence phenotype of *ore14/arf2* was also observed under artificially induced senescence conditions, we believe that this possibility is very low.

Role of ARF2 in auxin signalling

ARFs with a glutamine-rich MR might function as activators of auxin-responsive gene expression in transiently transfected protoplasts, while ARFs with proline- and/or serine-rich MRs, such as ARF1 or ARF2, repress the transcription of reporter genes under the control of synthetic AuxREs (Tiwari *et al.*, 2003). However, when compared with the global gene expression profiling of liquid-cultured *arf2* seedlings or the inhibition of root growth by exogenous auxin, no significant effect of the mutation was observed (Okushima *et al.*, 2005a). The fact that the *arf2* mutation does not affect auxin-induced or -repressed genes indicates that ARF2 does not participate in auxin signalling in a particular developmental stage or organ. However, in contrast to earlier reports, it was observed that the *ore14/arf2* mutant exhibited enhanced sensitivity to auxin, as assessed by the inhibition of hypocotyl growth. This implies that ARF2 functions as a transcription repressor of auxin-responsive gene expression, as previously proposed by Tiwari *et al.* (2003). The results seen here along with other data also suggest that ARF2-mediated auxin signalling is temporally and spatially regulated.

Other studies have shown that ARF2 links brassinosteroid (BR) and auxin biosynthetic pathways (Vert *et al.*, 2008). Thus, the effect of the *ore14/arf2* mutation on auxin responses might be due to an altered BR response. However, in the study of Vert *et al.* (2008) no difference in response to exogenous BR was detected between the wild type and the mutant, indicating that the *ore14/arf2* mutation does not cause defects in BR perception and response. Conceivably, an increased sensitivity to auxin conferred by the *arf2* mutation might not be due to an altered BR response.

ARF2 is a major player in the auxin-mediated control of leaf longevity

The role of auxin in leaf senescence has been elusive, particularly due to its involvement in various aspects of plant development. However, evidence that auxin has a role in suppressing leaf senescence has accumulated. Shoji *et al.* (1951) reported that auxin levels decline with leaf age. The exogenous application of auxin to leaves represses transcription of some senescence-associated genes (Noh and Amasino, 1999). Together, these results imply that

decreased auxin levels along with leaf age might induce the initiation and/or progression of leaf senescence, indicating that auxin is a negatively acting factor of leaf senescence. Genetic mutations that alter auxin signalling also support the involvement of auxin in controlling leaf senescence (Ellis *et al.*, 2005).

What is the major player in the auxin-mediated control of leaf senescence? ARFs regulate auxin-mediated transcriptional activation and repression, and each ARF protein is thought to play a central role in various auxin-mediated developmental processes. A few lines of our data, together with previous results, indicate that ARF2 might be a major player in the auxin-mediated control of leaf senescence. First, ARF2 affects many aspects of senescence, whether induced by age, darkness, hormones, or oxidative stress. Second, phenotypic analyses of T-DNA insertion lines for ARF family genes have revealed that ARF2 plays a major role in controlling leaf senescence and that ARF1 acts only in a partially redundant manner (Ellis *et al.*, 2005). Third, microarray analysis has shown that ARF2 transcripts increase in senescing leaves when induced by developmental ageing or darkness (Buchanan-Wollaston *et al.*, 2005; Ellis *et al.*, 2005). The ARF7 and ARF19 genes are also induced in senescing leaves (Lin and Wu, 2004). However, mutations in these genes do not alter the leaf senescence phenotype, although they enhance the delay in senescence conferred by the *arf2* mutation (Ellis *et al.*, 2005).

How then is leaf senescence delayed in the *arf2/mnt1/ore14* mutant? With the theory that auxin functions as a suppressor of leaf senescence and that ARF2 is a repressor of auxin signalling, it is conceivable that the reduced functioning of ARF2 in the mutant can cause less repression of auxin signalling with increased auxin sensitivity, leading to delayed senescence. Our data, as well as previous results, suggest that auxin has a role in suppressing leaf senescence, but suppression is decreased with leaf ageing, possibly through the increased activity of ARF2. Further studies on the identification of ARF2 downstream target genes or interacting proteins will help to dissect the senescence pathways involved in the ARF2-mediated control of leaf longevity.

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