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RESEARCH PAPER

Arabidopsis CPR5 is a senescence-regulatory gene with pleiotropic functions as predicted by the evolutionary theory of senescence

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Abstract

Evolutionary theories of senescence predict that genes with pleiotropic functions are important for senescence regulation. In plants there is no direct molecular genetic test for the existence of such senescence-regulatory genes. *Arabidopsis cpr5* mutants exhibit multiple phenotypes including hypersensitivity to various signalling molecules, constitutive expression of pathogen-related genes, abnormal trichome development, spontaneous lesion formation, and accelerated leaf senescence. These indicate that *CPR5* is a beneficial gene which controls multiple facets of the *Arabidopsis* life cycle. Ectopic expression of *CPR5* restored all the mutant phenotypes. However, in transgenic plants with increased *CPR5* transcripts, accelerated leaf senescence was observed in detached leaves and at late development around 50 d after germination, as illustrated by the earlier onset of senescence-associated physiological and molecular markers. Thus, *CPR5* has early-life beneficial effects by repressing cell death and insuring normal plant development, but late-life deleterious effects by promoting developmental senescence. As such, *CPR5* appears to function as a typical senescence-regulatory gene as predicted by the evolutionary theories of senescence.

Key words: *Arabidopsis*, *cell death*, CPR5/*OLD1, evolutionary senescence, hormones, leaf senescence.*

Introduction

In animal and evolutionary biology, senescence is defined as a decline in age-specific fitness components due to internal physiological deterioration (Rose, 1991). Studies on evolutionary senescence aim to address why the mortality rates of individuals increase with advancing age and vary within populations and among species. Based on research, mainly in the animal field, two major theories of evolutionary senescence have been developed and are widely acknowledged (Kirkwood and Austad, 2000). The Antagonistic Pleiotropy Theory points out that evolution acts to maximize reproductive fitness and will allow the existence of mutations that have beneficial effects for early-life survival and reproduction despite the fact that these mutations may have deleterious late-life effects to promote senescence. The Mutation Accumulation Theory is based on the observation that the force of natural selection diminishes with age and predicts that a mutation with deleterious late-life effects which lead to senescence is acceptable if it will allow the carrier to reproduce before death. These two theories suggest that two classes of mutations are responsible for senescence: those with beneficial early-life effects but deleterious late-life effects; and late-acting mutations with purely deleterious effects (Kirkwood and Austad, 2000).

In yeast and animal ageing paradigms, both types of gene action have been validated and genes involved in the insulin/IGF (insulin growth factor)-1 signalling, metabolic

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flux, and resistance to oxidative stress have been shown to be the important players for lifespan regulation (Sgro and Partridge, 1999; Guarente and Kenyon, 2000; Gems and Partridge, 2001; Kenyon, 2001; Arantes-Oliveira *et al*., 2002; Biesalski, 2002; Hughes *et al*., 2002; Tatar *et al*., 2003). In plants, the term senescence is prevalently used in a physiological context to describe a genetically controlled developmental programme that leads to the death of plant cells, tissues, organs, and whole plants. There is a debate as to whether studies on leaf senescence can validate the evolutionary theories of senescence in plants (Thomas, 2002). Senescence in the evolutionary sense is based on studies on individuals at the population and species levels. One doubt is whether this definition can be 'scaled down' to individual leaves. However, it has been argued that leaves have a clear lifespan and demographic features, and hence can be viewed as cohorts in a population (Bleecker, 1998). Furthermore, leaf senescence is marked by the massive mobilization and recycling of the assimilated nutrients in the senescing leaf, and hence considered to be essential for ensuring survivability of a species (Buchanan-Wollaston *et al*., 2003; Hopkins *et al*., 2007). Due to such a strong adaptive advantage, leaf senescence appears to violate the definition of evolutionary senescence that occurs in the absence of natural selection and is non-adaptive. This conflict might be reconciled by considering leaf senescence as a deleterious consequence of the selection for the traits that enable nutrient mobilization for the success of reproduction (Bleecker, 1998; Schippers *et al*., 2007).

A direct test of the evolutionary theories of senescence in plants is to characterize the functionality of senescence-regulatory genes. In an effort to isolate *Arabidopsis* mutants that exhibit altered ethylene (ET)-induced leaf senescence, *old1* mutants were obtained, which have two distinct alterations: accelerated age-regulated senescence and an enhanced ET response (Jing *et al*., 2002, 2005). Subsequent map-based cloning showed that *old1* is allelic to *cpr5*, which has been recovered in screens for mutants with enhanced pathogen defence responses (Bowling *et al*., 1997), abnormal trichome development (Kirik *et al*., 2001), and dark-induced leaf senescence (Yoshida *et al*., 2002). Thus, *CPR5* appears to be involved in multiple processes of plant growth and development and responses to biotic and abiotic stresses. The pleiotropic effects of *CPR5* make it an ideal candidate to test whether this gene is a senescence-regulatory gene as predicted by the evolutionary theories of senescence. Taking advantage of the availability of multiple *cpr5* mutants from different genetic backgrounds, *cpr5*-induced phenotypes were explored in detail. Transgenic plants with increased *CPR5* mRNA levels were constructed to examine the effects of *CPR5* during late life, especially during plant senescence. It was shown that *CPR5* ensures normal growth and development by controlling hormonal signalling and by repressing cell death in early plant life. However, *CPR5* can promote leaf senescence in late development. These results are consistent with the notion that *CPR5* may be a senescence-regulatory gene as predicted by the Antagonistic Pleiotropy Theory of Evolutionary Senescence.

Materials and methods

Plant materials and growth conditions

Arabidopsis thaliana accessions L*er*-0 and Col-0 were the wild types. The mutant alleles and transgenic plants used were *old1-1* (renamed *cpr5-l1* in this paper) (Jing *et al*., 2002), *cpr5-l2*, *cpr5-l3*, *cpr5-1* (Bowling *et al*., 1997), *cpr5-2* (Boch *et al*., 1998), *hys1-1* (Yoshida *et al*., 2002), *ein2-1* (Guzman and Ecker, 1990), *ctr1-1* (Kieber *et al*., 1993), *jar1-1* (Staswick *et al*., 1992), and *abi4-1* (Finkelstein *et al*., 1998). In order to compare the effect of different *cpr5* mutations on various phenotypes, the *cpr5-l1* allele was crossed with Col-0, and *cpr5-l1/C* was selected from an F_2 population and thus contains a mixed L*er*-0 and Col-0 background.

Plants were grown in an organic-rich soil (TULIP PROFI No. 4, BOGRO B.V., Hardenberg, The Netherlands) or in Murashige and Skoog (MS) medium containing 0.8% agar under the conditions described by Jing *et al*. (2002).

Map-based cloning, complementation test, and construction of transgenic lines

cpr5-l1 was originally placed ~3 cM south of the single nucleotide polymorphism (SNP) marker SGCSNP84 at the bottom of chromosome 5. To perform fine mapping, 2000 F_2 *cpr5-l1* seedlings were selected from a mapping population generated by crossing *cpr5-l1* with Col-0. DNA was isolated using the SHORTY quick preparation method (http://www.biotech.wisc.edu/Arabidopsis). By comparing the genomes of Col-0 (TAIR database) and Cereon L*er*-0 (Monsanto SNPs and L*er*) (http://www.arabidopsis.org; Jander *et al*., 2002), potential SNPs were selected. Primers were designed, using the WebSNAPER program, that specifically amplified Col-0 DNA fragments, and used for PCR (Drenkard *et al*., 2000; http://ausubellab.mgh.harvard.edu/resources). The mutation was mapped onto a 15 kb region spanning three open reading frames including *CPR5*. Sequence analyses revealed a single nucleotide change inside *CPR5*. The other two L*er*-0 *cpr5* alleles were subsequently sequenced (Table 1). *Agrobacterium*-mediated transformation was performed to confirm further the identity of *old1* as a *cpr5* allele.

For constructing *CPR5* transgenic plants, full-length *CPR5* cDNA was amplified using primers designed either with or without an in-frame fusion of the HA epitope tag (YPYDVPDYA) and cloned behind a modified 35S CaMV promoter in the plant transformation vector pBI1.4T. All constructs were verified by sequencing and subsequently electroporated into *Agrobacterium tumefaciens* strain *GV3101*. The resulting bacteria were used to transform wild-type Col-0 (Clough and Bent, 1998). Transformants were selected on MS media containing 50 μg ml^{-1} kanamycin

Table 1. cpr5 *alleles identified or studied in this paper*

Mutant	Accession	Nucleotide change	AA change
cpr5-l1	L*er*-0	GGT to AGT	$459^{G\ to\ S}$
cpr5-l2	L*er*-0	TGG to TGA	$391^{W\ to\ stop}$
cpr5-l3	L*er*-0	GGG to GAG	$120^{G\ to\ D}$
cpr5-2	Col-0	TGG to TAG	$473^{W\ to\ stop}$
hys1-1	Col-0	TGG to TGA	$477^{W\ to\ stop}$

(Murashige and Skoog, 1962). Lines homozygous for single insertion events were used in further experiments.

Hormonal sensitivity assay

For ET sensitivity, seedlings were grown on MS media containing 1 μM ACC (1-aminocyclopropane-1-carboxylic acid) in the dark for 5 d, and the triple response was observed (Guzman and Ecker, 1990). Sugar sensitivity was determined by growth for 5 d in darkness on MS medium containing 1% sucrose. The hypocotyl lengths of the seedlings were subsequently compared (Dijkwel *et al*., 1997). The effect of JA on the inhibition of root elongation of light-grown seedlings was examined as described (Staswick *et al*., 1992). Briefly, seeds were germinated in light in vertical plates containing MS medium, 0.5% sucrose, and 20 μM MeJA, and the root elongation of 7-d-old light-grown seedlings was examined. A low concentration of sucrose (0.5%) was used to minimize the inhibiting effect of sugar. For ABA sensitivity, seeds were germinated in light on vertical plates containing MS medium, 0.5% sucrose, and 0.3 μM ABA. The growth of 7-d-old light-grown seedlings was examined. Seedlings and detached leaves were incubated in a growth chamber of 22 °C and, when grown in light, the intensity was ~60 $\mu molm^{-2}\ s^{-1}$.

Chlorophyll content measurement and gene expression analysis

For chlorophyll content measurement and northern blotting, rosette leaf samples were collected from 30-d-old soil-grown *Arabidopsis* plants. For northern blotting, whole rosettes were used. Leaf samples were prepared and analysed as described by Jing *et al*. (2002).

For real-time PCR measurements of *CPR5* mRNA levels, approximately 150 mg of tissue was harvested from 3-week-old soil-grown wild-type Col-0, mutant *cpr5-1*, and wild-type plants overexpressing *CPR5* cDNA (*C5-7*, *C4-3*, and *C8-6*) or HA-tagged *CPR5* cDNA (*N5-6*). Subsequently, total RNA was extracted as described by Cao *et al*. (1997). Ten micrograms of RNA was treated with DNase I according to the manufacturer's instructions (Ambion Inc., Austin, TX, USA). One microgram of RNA was incubated with Superscript II reverse transcriptase and ligo(dT) in a 20 μl reaction to synthesize cDNA (Invitrogen, Carlsbad, CA, USA). For the quantitative PCR, 2 μl of the cDNA product was used as template with the *CPR5*-specific primers, whereas 2 μl of a 20-fold dilution was used for reactions with *Ubiquitin5* (*UBQ5*, At3g62250.1)-specific primers. The final primer concentration in all reactions was 0.5 μM. Quantitative PCR was carried out using the SYBR green PCR kit (QIAgen, Valencia, CA, USA) and a Roche Lightcycler real-time PCR machine according to the manufacturer's instructions (Roche, Mannheim, Germany). The relative number of *CPR5*-specific transcripts was determined in three replicate experiments by normalization to *UBQ* transcript levels.

HPLC-MS (high pressure liquid chromatograph–mass spectrometry) analyses of salicylic acid (SA) and jasmonic acid (JA)

Rosette leaves numbers 3 and 4 without any signs of visible yellowing were taken from 21-d-old soil-grown plants and used to measure the SA and JA concentrations according to a procedure derived from Wilbert *et al*. (1998). Briefly, ~200 mg of leaf tissues were ground in liquid nitrogen into fine powder and extracted with 500 μl of acidified MeOH (methanol with 0.1% concentrated HCl) overnight at 4 °C. After centrifugation, the supernatant was collected, diluted to 35% with water, and centrifuged before injecting 100 μl of it into HPLC coupled on-line with a mass spectrometer for quantification. The injection was done with a Perkin-Elmer series 200 autosampler. Before and after injection the injector and the needle were flushed twice with 0.1% NH_4OH in 50% MeOH to remove the residual SA or JA. MeOH (gradient grade), formic acid (p.a., 98–100%) and ammonia solution (p.a., 25%) were purchased from Merck, Darmstadt.

For HPLC, both JA and SA were negatively charged by post-column addition of 1% NH_4OH solution in MeOH with a flow of 100 $\mu l\ min^{-1}$, delivered by a Kratos spectroflow 400-pump. JA and SA were separated under acidic conditions by running a gradient of aqueous 0.1% formic acid and 0.1% formic acid in MeOH over a 2.1 mm column (Alltech Alltima C18 5μm). The gradient was delivered by two Perkin-Elmer series 200 LC pumps at a flow rate of 200 $\mu l\ min^{-1}$, started with 30% MeOH for 1 min, raised to 95% in 5 min, and retained for 5 min, then dropped to 30% in 2 min. A 6 min interval was used for equilibration.

For mass spectrometry, the free acids JA and SA were analysed in the negative ion-mode by measuring a small range in a Q1-profile-scan-mode combined with 'up-front' collision to see the M-44-ion of SA (loss of CO_2) and to avoid association of the formate-ion with JA (M+45). The M-44-ion of JA was not observed. The MS system consisted of an API3000 mass spectrometer (Applied Biosystems/MDS-SCIEX, Toronto, Canada) and a triple quadrupole mass spectrometer equipped with a Turbo Ion-spray interface. The 200 $\mu l\ min^{-1}$ HPLC flow, combined with a 100 $\mu l\ min^{-1}$ post-column flow, were introduced through the ion-spray interface with the temperature of the heater set to 450 °C. The state file was as follows: NEB (zero air)=14, CUR=14, IS= –4500, TEM=450, OR= –50, RNG= –200, Q0=11, IQ1=11, St=15, RO1=11, IQ2=20, RO2=100, St3=120, RO3-102, DF=300, CEM=2500. For SA, the range of 136–139 amu and 92–95 amu with a step-size of 0.100 amu and a dwell-time of 1 ms was analysed. The molecular weight of SA is 138. The M-1-ion is m/z 137 and the M-1-44 is m/z 93. For JA, the range of 208–211 amu with the same step-size and dwell-time was analysed. The molecular weight of JA is 210. The M-1-ion is m/z 209. SIM was avoided to double-check the isotope patterns of the free acids. Due to the interference of many unknown products, slight shifting of the retention time for the same ions was observed. The area under the ion-signals was calculated with MacQuan 1.7 (PE SCIEX). To confirm the authenticity, SA and JA standards were added to the plant extracts as controls.

Results

Identification of Ler*-0 alleles of* cpr5

Three *old1* alleles were isolated in a screen for leaf senescence mutants that showed accelerated visible yellowing upon exposure to 10 $\mu l\ l^{-1}$ ET for 3 d; the details of this screen were reported previously (Jing *et al*., 2002). The *OLD1* gene was cloned by map-based cloning as described in Materials and methods, and complementation tests showed that *old1* mutants are allelic to *cpr5* (data not shown). Therefore, the *old1-1*, *old1-2*, and o*ld1-3* alleles were renamed as *cpr5-l1*, *cpr5-l2*, and *cpr5-l3*, respectively (Table 1).

Mutations in CPR5 *induce defects in multiple developmental processes*

The responses of *cpr5* mutants to multiple stimuli involved in plant growth and development and stress responses such as hormones and sugar were examined.

To examine the potential effects of different genetic backgrounds on *cpr5* mutations, the *cpr5-l1/C* line was generated as described in Materials and methods. Thus, the *cpr5-l1/C* line contains the *cpr5-l1* mutation with a mixed Col-0/L*er* background. Regardless of genetic background and the nature of mutations, all the examined *cpr5* mutants are hypersensitive to ET and sugar (Fig. 1A–C), which is consistent with previous reports (Jing *et al*., 2002; Yoshida *et al*., 2002). The root growth of *cpr5* seedlings was strongly inhibited in media containing low levels of exogenously applied ABA and MeJA (Fig. 1D, E). Thus, *cpr5* mutants exhibit enhanced responses to ET, sugar, JA, and ABA, indicating that *CPR5* is involved in seedling growth and development by controlling responses to multiple signalling pathways.

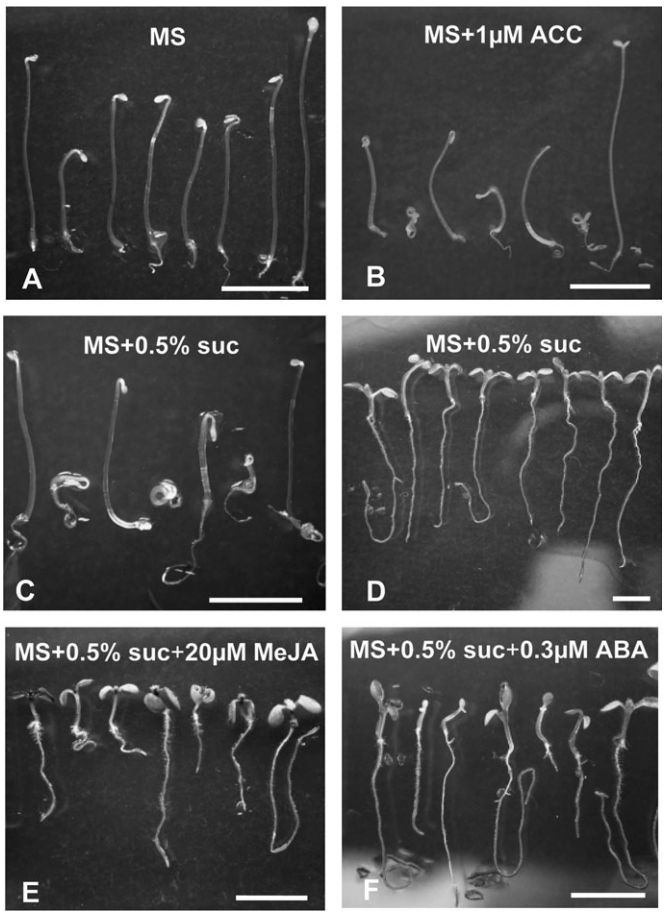

Fig. 1. Enhanced responses of *cpr5* mutants to various signalling molecules. (A–C) Five-day-old seedlings grown in darkness on MS (A), MS supplemented with 1 μM ACC (B), or 0.5% sucrose (C). The first six seedlings are L*er*-0, *cpr5-l1*, Col-0, *cpr5-2*, *hys1-1*, and *cpr5-l1/C*, and to the right are *abi4-1* and *ein2-1* (A), *ein2-1* (B), and *abi4-1* (C). (D–F) Seven-day-old seedlings grown under light on MS and 0.5% sucrose (D), MS and 0.5% sucrose supplemented with 20 μM MeJA (E), or MS and 0.3 μM ABA (F). The first six seedlings are L*er*-0, *cpr5-l1*, *cpr5-l1/C*, Col-0, *cpr5-2*, and *hys1-1*, and to the right are *jar1-1* and *abi4-1* (D), *jar1-1* (E), and *abi4-1* (F). The experiments were repeated at least three times with similar results, and representative seedlings are shown. White bars represent 0.5 cm.

It was reported previously that SA accumulates to a substantially high level in *cpr5* mutants (Bowling *et al*., 1997; Clarke *et al*., 2000, 2001; Jirage *et al*., 2001). The endogenous levels of several hormones in *cpr5* mutants were then quantified to test the hypothesis that the altered responses may be associated with the alteration of hormonal production. Figure 2 shows that besides SA, the *cpr5* mutants had increased JA levels as well. The ET production was also measured in etiolated *cpr5* mutants and the results showed that *cpr5* mutants had a wild-type ET production level (data not shown). Thus, *cpr5* mutations impose different effects on SA, JA, and ET production in *Arabidopsis*.

Mutations in *CPR5* also cause precocious cell death in the form of spontaneous lesions and senescence (Fig. 3A). This was reflected by decreases in leaf chlorophyll contents (Fig. 3B). Analyses of the expression profiles of senescence-associated genes showed that *cpr5* mutants similarly exhibited earlier and higher transcription levels of *SAG12*, *SAG13*, *SAG14*, and *SAG21* in comparison with wild-type plants. *PR-1* mRNA levels were also higher in *cpr5* mutants (Fig. 3C). Thus, mutations in *CPR5* result in precocious developmental cell death.

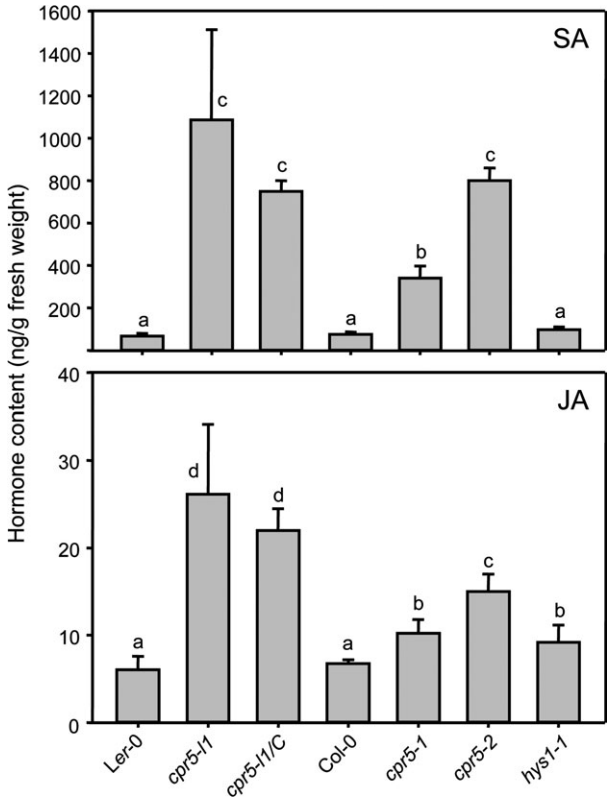

Fig. 2. Endogenous SA and JA levels in wild-type and *cpr5* mutant plants. Rosette leaves 3 and 4 from 21-d-old soil-grown seedlings were used for HPLC-MS quantification as detailed in Materials and methods. Lines with the same letters are not significantly different from each other at the α=0.05 significance level as determined by Duncan's Multiple Variant Mean Test. For each line, at least four replicates were used for quantification.

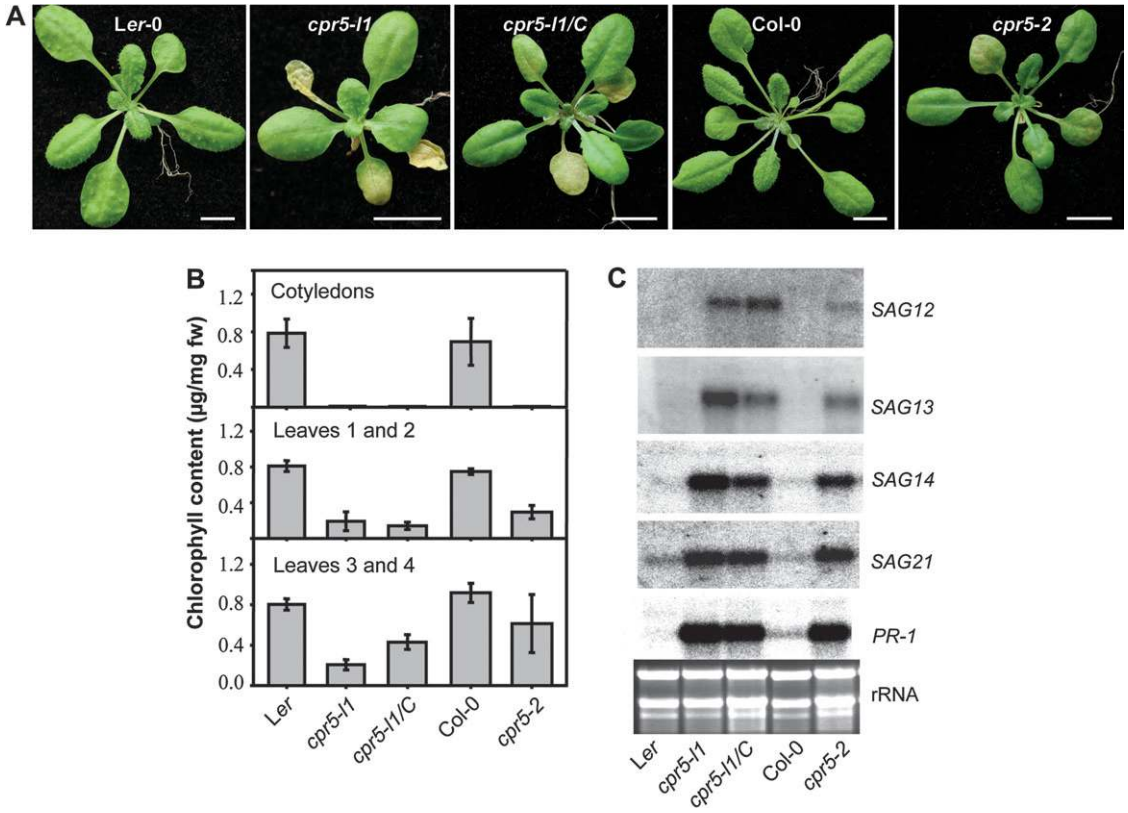

Fig. 3. The senescence syndrome of *cpr5* mutants. (A) Plants were grown on soil under the conditions described in Materials and methods for 30 d and representative plants were selected and photographed; (B) the chlorophyll contents were quantified and shown as mean ±SD of at least four replicates; and (C) the abundance of the mRNA of various genes analysed by RNA gel blot analysis.

Overexpression of CPR5 *restores all the mutant phenotypes*

Utilizing the transcriptome data on the Genevestigator website (Zimmermann *et al*., 2004), the transcription profiles of *CPR5* during development were examined. As shown in Fig. 4, *CPR5* is constitutively expressed during the *Arabidopsis* life cycle. The *CPR5* transcripts increase towards the end of development at age 45–50, which is characteristic of senescence-associated genes. Interestingly, the transcripts of two classical *SAG*s, *SAG12* and *SAG13*, increase two to three developmental stages earlier than *CPR5*, indicating that the induction of *SAG12* and *SAG13* expression during senescence may not be associated with the induction of *CPR5* expression.

The expression pattern of *CPR5* indicates that it may have a role in promoting senescence during late development only, while its function in the regulation of the onset of senescence during early development may be limited. To test this hypothesis, transgenic plants expressing various amounts of *CPR5* transcripts were constructed as described in Materials and methods. Four representative transgenic lines were used in this study. Quantitative PCR measurements indicated that lines *C5-7*, *C4-3*, and *N5-6* had higher than wild-type *CPR5* mRNA levels, whereas line *C8-6* and *cpr5-1* mutants had lower transcript levels (Fig. 6B). As shown in Fig. 5, the *C8-6* plants displayed all the *cpr5*-mutant phenotypes such as enhanced hormonal sensitivity, reduced leaf and plant size, and abnormal trichome development, consistent with knocked-down *CPR5* transcription. By contrast, in the *C5-7, C4-3*, and *N5-6* plants, *cpr5* mutant phenotypes were not observed. Thus, overexpression of *CPR5* does not affect early seedling and plant development.

CPR5 *overexpression accelerates senescence at late development*

Senescence phenotypes were examined in the transgenic lines and in the *cpr5-1* mutant. Leaf senescence was induced by placing detached leaves in continuous light for 7 d. Figure 6A shows the results of the detachment-induced senescence experiment. As would be expected, the *cpr5-1* mutant and line *C8-6* contained less chlorophyll in comparison with the wild type. The chlorophyll contents in the overexpression lines *C5-7*, *C4-3*, and *N5-6* were lower than that of the wild type, but were higher than those of the *cpr5-1* mutant and *C8-6* plants. Similar results were obtained when the detached leaves were incubated with JA or ABA (data not shown). Thus, leaves of the transgenic lines exhibited accelerated drops in chlorophyll content upon detachment, demonstrating that

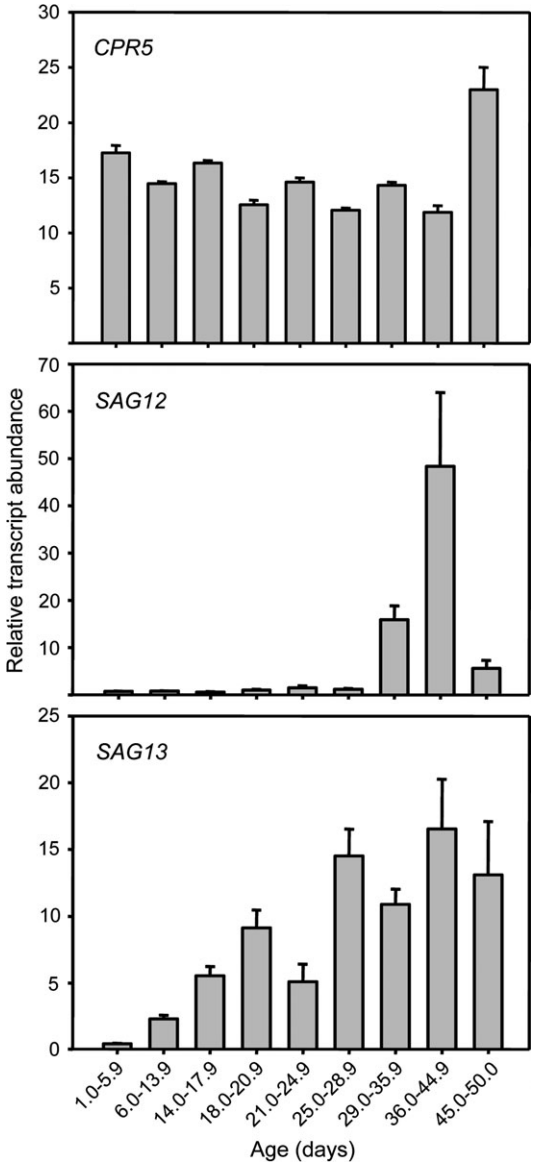

Fig. 4. *CPR5*, *SAG12*, and *SAG13* mRNA levels during development. Data of the mRNA levels during development were obtained from Genevestigator and plotted.

increasing *CPR5* transcripts promote senescence in detached leaves.

Developmental senescence was further characterized. At ~40 d after germination visible yellowing was observed both in the wild type and the transgenic lines (Fig. 6C), but chlorosis proceeded faster and occurred in more leaves in the overexpression lines (Fig. 6D). The correlation between *CPR5* expression and the advanced senescence syndrome was studied further using molecular markers (Fig. 6E). RNA gel blot analysis showed that at this developmental stage, the *CPR5* mRNA level was highest in *C5-7*, slightly lower in *N5-6*, and comparable with the wild type in *C4-3*. There was a good correlation between the *CPR5* and *SAG12* mRNA levels; the low *CPR5* levels in *cpr5-1* and *C8-6* correlated with appreciable *SAG12* expression, whereas high *CPR5* transcript levels in *C5-7* and *N5-6* correlated with increased *SAG12* mRNA levels. *SAG12* mRNA was not detected in the wild-type Col-0 tissue. The results suggest that increasing *CPR5* levels cause accelerated senescence at late developmental stages. The general defence response marker *PR-1* mRNA levels were also enhanced in the transgenic lines and the *cpr5-1* mutant.

Taken together, plants with reduced *CPR5* mRNA levels had phenotypes similar to *cpr5* mutants, whereas *CPR5* overexpression only caused early leaf senescence during later stages of plant development.

Discussion

It has been argued that a plant or animal genome is optimized for early survival and reproduction and that senescence is a consequence of such genome optimization (de Magalhaes and Church, 2005; Schippers *et al*., 2007). At biochemical and molecular levels leaf senescence resembles animal ageing in various aspects. In most cases the strategies used by plants to regulate senescence are similar to those in animals (Gan, 2003; Jing *et al*., 2003; Lim *et al*., 2003). Leaf senescence is marked by changes in gene expression profiles. Many senescence-associated genes (*SAG*s) have been isolated and shown to include genes involved in protein and lipid degradation, transport, cellular stress- and defence-related responses, transcriptional regulation, and signalling pathways (Buchanan-Wollaston, 1997; Nam, 1997; Quirino *et al*., 2000; Chen *et al*., 2002; Buchanan-Wollaston *et al*., 2003). In ageing yeast and animals, similar groups of genes displayed such senescence-associated changes in their expression profiles (Zou *et al*., 2000; Weindruch *et al*., 2001; Pletcher *et al*., 2002; Kyng *et al*., 2003). Thus, similar molecular and cellular processes may take place during leaf senescence and animal ageing, which argues that genes regulating leaf senescence and animal ageing may be similar in nature.

Here this issue is addressed by studying the functions of *CPR5* during development. The results show that *CPR5* is a senescence-regulatory gene with pleiotropic functions as predicted by the evolutionary theories of senescence, which were developed from studying animal ageing.

CPR5 has early-life beneficial effects

The *cpr5* mutants have many phenotypes and were recovered in several mutant screens, indicative of the involvement of *CPR5* in various aspects of the plant life cycle. The beneficial effects of *CPR5* are illustrated by the following observations. (i) Seedlings carrying *cpr5* alleles were found to be hypersensitive to ET, sugar, JA, and ABA responses (Jing *et al*., 2002; Yoshida *et al*., 2002;

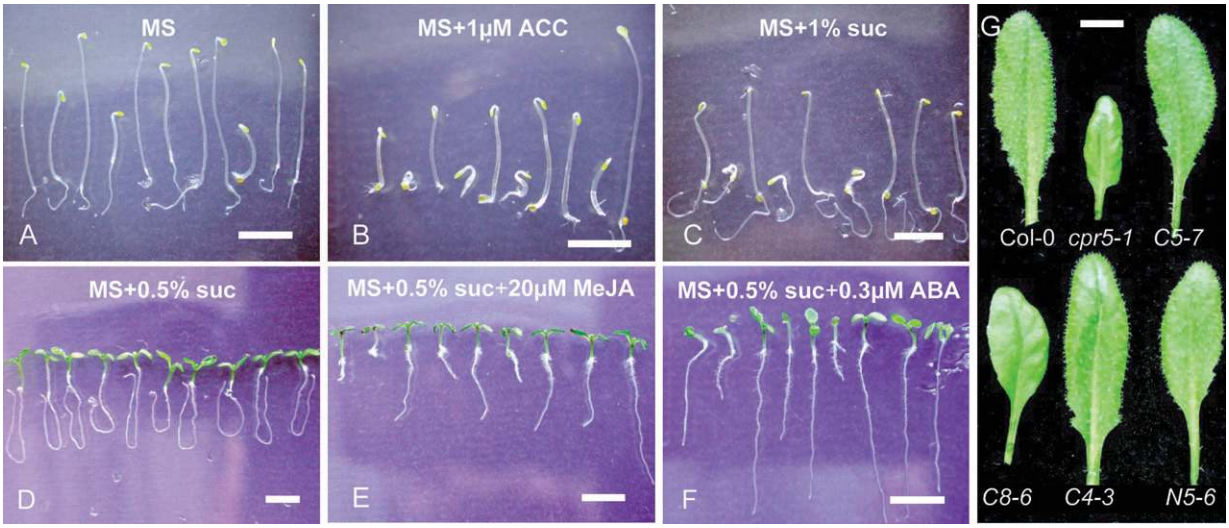

Fig. 5. Characterization of hormonal sensitivity and growth of *CPR5* transgenic lines, *cpr5* mutants, and wild type. (A–F) The first eight seedlings are L*er*-0, *cpr5-1l*, Col-0, *cpr5-1*, *C5-7*, *C8-6*, *C4-3*, and *N5-6*, and the rest are *ctr1-1*, *ein2*, and *abi4-1* (A), *ctr1-1* and *ein2* (B), *abi4-1* (**C**), *jar1-1* and *abi4-1* (D), *jar1-1* (E), and *abi4-1* (F). Seedlings were grown on plates containing the components indicated in darkness for 5 d (A–C) or in light for 7 d (D–F). (G) Representative mature leaves from the lines indicated. White bars represent 0.5 cm.

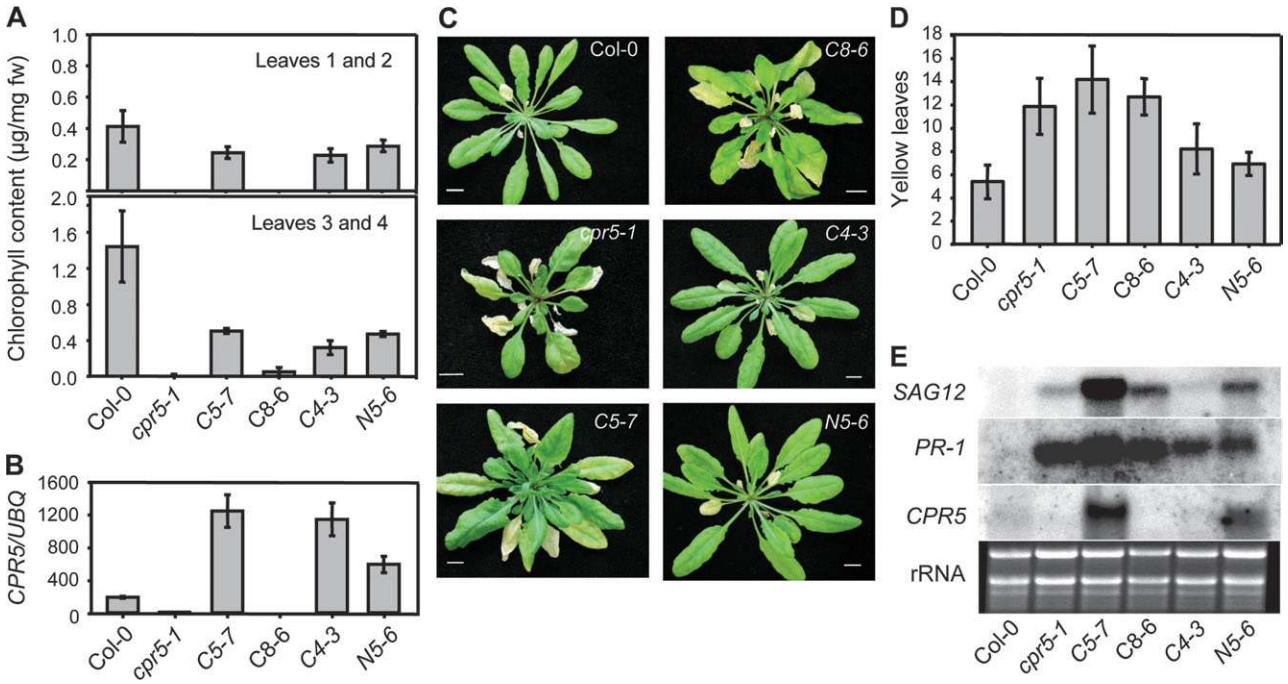

Fig. 6. Characterization of the senescence syndrome in *cpr5* mutants, *CPR5* transgenic lines, and the wild type. (A) Comparison of detachment-induced senescence between wild type, *cpr5-1* mutants, and *Arabidopsis* transgenic lines with varied *CPR5* mRNA levels. Shown are the chlorophyll contents of the first and second pairs of rosette leaves detached from 21-d-old soil-grown plants. Leaves were incubated in light on two layers of Whatman filter papers saturated with MES solution (pH 5.7) for 7 d and collected for chlorophyll content measurement. Four replicates of two pairs of leaves were analysed for each line. Results are shown as mean ±SD. (B) Quantification of *CPR5* mRNA levels of *Arabidopsis* transgenic lines, the *cpr5-1* mutant, and the wild type. The abundance of *CPR5* relative to ubiquitin (*UBQ*) mRNA, expressed as mean ±SD, is shown. (C, D) Comparison of *in planta* senescence of *CPR5* transgenic lines, the *cpr5-1* mutant, and the wild type. Plants were grown on soil for 50 d and the number of yellow leaves was recorded as described in Materials and methods. (C) Representative plants whose inflorescence stems were removed are shown. (D) The results of visible yellowing quantification that are presented as mean ±SD from observations on at least 10 plants per line are shown. (E) RNA gel blot analysis showing the abundance of the mRNA of various genes in *CPR5* transgenic lines, the *cpr5-1* mutant, and the wild type. Rosette leaves 1–6 were harvested from 50-d-old soil-grown plants for total RNA isolation, and 10μg of total RNA was used for RNA gel blot analysis. ^{32}P-labelled probes were used. The rRNA picture is shown as a loading control.

this study). These signalling molecules all contribute to seedling growth and development in various ways (Creelman and Mullet, 1997; Johnson and Ecker, 1999; Rolland *et al*., 2002). Thus, it appears that *CPR5* controls normal seedling growth and development by mediating responses to multiple hormonal and sugar signalling components. (ii) Adult *cpr5* mutants have a stunted plant status and reduced trichome development. The *cpr5* mutants also produce fewer seeds and a smaller rosette under various conditions (Heidel *et al*., 2004). Thus, *cpr5* mutations reduce the fitness of adult plants. (iii) Overexpression of *CPR5* restored all the phenotypes induced by *cpr5* mutations, and transgenic lines with increased *CPR5* transcripts displayed normal growth and development. The reduced growth and development of adult *cpr5* plants could be caused by a number of factors. They could be due to the enhanced responses to the aforementioned multiple signalling molecules and/or elevated SA and JA levels. They could also result from the occurrence of precocious cell death as envisaged by lesion formation and accelerated senescence. Whatever the reasons are, *CPR5* appears to be essential for *Arabidopsis* to develop into a normal adult plant. These observations clearly demonstrate that *CPR5* has predominant early-life beneficial effects.

CPR5 *has late-life deleterious effects*

CPR5 transcripts increase during late developmental stages, which is consistent with a role in promoting senescence. This is in agreement with observations that *CPR5* levels increased in mature leaves of transgenic plants harbouring *CPR5* promoter:GUS reporter constructs (H-C Jing *et al*., unpublished results). Construction and study of *CPR5* overexpression lines provides important clues to the function of *CPR5* during late *Arabidopsis* life. The examined transgenic lines with constitutively enhanced *CPR5* transcripts exhibited accelerated leaf senescence, both upon detachment and *in planta*. This demonstrates that *CPR5* has late-life deleterious effects. It is important to note that the timing of senescence in *CPR5* overexpression lines is different from that of *cpr5* mutants. In *CPR5* transgenic lines, senescence only occurs after reproduction (bolting), whereas in *cpr5* mutants senescence starts in young plants before reproduction. In this sense, *CPR5* represses cell death before reproduction and promotes cell death after reproduction.

Evolution of senescence-regulatory genes in plants

The results obtained in this study showed that *CPR5* differentially exerts its functions during *Arabidopsis* growth and development. *CPR5* exhibits early-life beneficial effects by ensuring normal seedling growth and repressing cell death in adult plants. However, after reproduction a functional *CPR5* may promote 'normal' senescence and hence is deleterious. Such a separation of the functions of *CPR5* throughout development mimics the action of the insulin/IGF-1 signalling pathway and *p53* in animal and human cells (Zafon, 2007). The insulin/IGF-1 signalling pathway has pleiotropic functions and is shown to employ independent mechanisms to regulate lifespan and reproduction (Dillin *et al*., 2002). p53 is a genome guardian; the deficiency of p53 proteins leads to cancer and tumour development due to increased cellular damage, suggesting that p53 has clear early-life beneficial effects (Levine, 1997; Sharpless and DePinho, 2002). Nonetheless, a p53 mutant mouse line ($p53^{+/m}$), in which the stability and activities of the wild-type p53 protein were augmented in the presence of a mutant allele, developed fewer tumours than wild-type ($p53^{+/+}$) homozygotes, but exhibited faster ageing (Tyner *et al*., 2002). Clearly, maintaining a higher p53 level in late life is deleterious. Thus, *CPR5* seems to function as a typical senescence-regulatory gene as predicted by the evolutionary theory of senescence. However, at the DNA and protein levels, *CPR5* shares no similarities with any genes in the insulin/IGF-1 signalling pathway, or p53, in agreement with the notion that, although plants may use similar strategies to control senescence, the particular molecular mechanisms can be different (Jing *et al*., 2003). Further molecular genetic and biochemical studies that unravel how CPR5 works in a plant cell will allow a better comparison of senescence-regulatory mechanisms across kingdoms.

So far, the cloned senescence-regulatory genes in plants share a common feature, which is that they are involved in plant growth and development as well. For instance, *ORE9* encodes an F-box protein and is part of the ubiquitination protein degradation machinery (Woo *et al*., 2001). *ore9*/*max2* alleles were also recovered in a screen for mutants with altered shoot lateral branching (Stirnberg *et al*., 2002). *SAG101* was shown to be involved in lipid metabolism (He and Gan, 2002), and *ORE4* encodes the plastid ribosomal small subunit protein 17 (*PRPS17*) that is important for protein synthesis (Woo *et al*., 2002). Furthermore, a recent characterization of the *SGR* gene in rice indicates the possible existence of genes with deleterious effects late in life (Park *et al*., 2007). However, it is not clear whether these genes are the *sensu stricto* senescence-regulatory genes as defined by the evolutionary theories of senescence. It is hoped the study presented here will stimulate more research to address this issue.

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References

Arantes-Oliveira N, Apfeld J, Dillin A, Kenyon C. 2002. Regulation of life-span by germ-line stem cells in *Caenorhabditis elegans*. *Science* **295,** 502–505.

Biesalski HK. 2002. Free radical theory of ageing. *Current Opinion in Clinical Nutrition and Metabolic Care* **5,** 5–10.

Bleecker AB. 1998. The evolutionary basis of leaf senescence: method to the madness? *Current Opinion in Plant Biology* **1,** 73–78.

Boch J, Verbsky ML, Robertson TL, Larkin JC, Kunkel BN. 1998. Analysis of resistance gene-mediated defense responses in *Arabidopsis thaliana* plants carrying a mutation in *CPR5*. *Molecular Plant–Microbe Interactions* **11,** 1196–1206.

Bowling SA, Clarke JD, Liu Y, Klessig DF, Dong X. 1997. The *cpr5* mutant of Arabidopsis expresses both NPR1-dependent and NPR1-independent resistance. *The Plant Cell* **9,** 1573–1584.

Buchanan-Wollaston V. 1997. The molecular biology of leaf senescence. *Journal of Experimental Botany* **48,** 181–199.

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T, Pink D. 2003. The molecular analysis of leaf senescence – a genomic approach. *Plant Biotechnology Journal* **1,** 3–22.

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X. 1997. The Arabidopsis *NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. *Cell* **88,** 57–63.

Chen W, Provart NJ, Glazebrook J, *et al*. 2002. Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. *The Plant Cell* **14,** 559–574.

Clarke JD, Aarts N, Feys BJ, Dong X, Parker JE. 2001. Constitutive disease resistance requires *EDS1* in the Arabidopsis mutants *cpr1* and *cpr6* and is partially *EDS1*-dependent in *cpr5*. *The Plant Journal* **26,** 409–420.

Clarke JD, Volko SM, Ledford H, Ausubel FM, Dong X. 2000. Roles of salicylic acid, jasmonic acid, and ethylene in *cpr*-induced resistance in Arabidopsis. *The Plant Cell* **12,** 2175–2190.

Creelman RA, Mullet JE. 1997. Biosynthesis and action of jasmonates in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **48,** 355–381.

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The Plant Journal* **16,** 735–743.

de Magalhaes JP, Church GM. 2005. Genomes optimize reproduction: aging as a consequence of the developmental program. *Physiology* **20,** 252–259.

Dijkwel PP, Huijser C, Weisbeek PJ, Chua NH, Smeekens SCM. 1997. Sucrose control of phytochrome A signalling in Arabidopsis. *The Plant Cell* **9,** 583–595.

Dillin A, Crawford DK, Kenyon C. 2002. Timing requirements for insulin/IGF-1 signalling in *C. elegans*. *Science* **298,** 830–834.

Drenkard E, Richter BG, Rozen S, Stutius LM, Angell NA, Mindrinos M, Cho RJ, Oefner PJ, Davis RW, Ausubel FM. 2000. A simple procedure for the analysis of single nucleotide polymorphisms facilitates map-based cloning in Arabidopsis. *Plant Physiology* **124,** 1483–1492.

Finkelstein RR, Wang ML, Lynch TJ, Rao S, Goodman HM. 1998. The Arabidopsis abscisic acid response locus *ABI4* encodes an APETALA 2 domain protein. *The Plant Cell* **10,** 1043–1054.

Gan S. 2003. Mitotic and postmitotic senescence in plants. *Science of Aging Knowledge Environment* Issue **38,** p.re7.

Gems D, Partridge L. 2001. Insulin/IGF signalling and ageing: seeing the bigger picture. *Current Opinion in Genetics & Development* **11,** 287–292.

Guarente L, Kenyon C. 2000. Genetic pathways that regulate ageing in model organisms. *Nature* **408,** 255–262.

Guzman P, Ecker JR. 1990. Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *The Plant Cell* **2,** 513–523.

He Y, Gan S. 2002. A gene encoding an acyl hydrolase is involved in leaf senescence in Arabidopsis. *The Plant Cell* **14,** 805–815.

Heidel AJ, Clarke JD, Antonovics J, Dong X. 2004. Fitness costs of mutations affecting the systemic acquired resistance pathway in *Arabidopsis thaliana*. *Genetics* **168,** 2197–2206.

Hopkins M, Taylor C, Liu Z, Ma F, McNamara L, Wang T-W, Thompson JE. 2007. Regulation and execution of molecular disassembly and catabolism during senescence. *New Phytologist* **175,** 201–214.

Hughes KA, Alipaz JA, Drnevich JM, Reynolds RM. 2002. A test of evolutionary theories of ageing. *Proceedings of the National Academy of Sciences, USA* **99,** 14286–14291.

Jander G, Norris SR, Rounsley SD, Bush DF, Levin IM, Last RL. 2002. Arabidopsis map-based cloning in the post-genome era. *Plant Physiology* **129,** 440–450.

Jing HC, Hille J, Dijkwel PP. 2003. Ageing in plants: conserved strategies and novel pathways. *Plant Biology* **5,** 455–464.

Jing HC, Sturre MJG, Hille J, Dijkwel PP. 2002. Arabidopsis *onset of leaf death* mutants identify a regulatory pathway controlling leaf senescence. *The Plant Journal* **32,** 51–63.

Jing HC, Schippers JHM, Hille J, Dijkwel PP. 2005. Ethylene-induced leaf senescence depends on age-related changes and *OLD* genes in Arabidopsis. *Journal of Experimental Botany* **56,** 2915–2923.

Jirage D, Zhou N, Cooper B, Clarke JD, Dong X, Glazebrook J. 2001. Constitutive salicylic acid-dependent signalling in *cpr1* and *cpr6* mutants requires *PAD4*. *The Plant Journal* **26,** 395–407.

Johnson PR, Ecker JR. 1999. The ethylene gas signal transduction pathway: a molecular perspective. *Annual Review of Genetics* **32,** 227–254.

Kenyon C. 2001. A conserved regulatory system for ageing. *Cell* **105,** 165–168.

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR. 1993. *CTR1*, a negative regulator of the ethylene response pathway in Arabidopsis, encodes a member of the raf family of protein kinases. *Cell* **72,** 427–441.

Kirik V, Bouyer D, Schobinger U, Bechtold N, Herzog M, Bonneville JM, Hulskamp M. 2001. *CPR5* is involved in cell proliferation and cell death control and encodes a novel transmembrane protein. *Current Biology* **11,** 1891–1895.

Kirkwood TB, Austad SN. 2000. Why do we age? *Nature* **408,** 233–238.

Kyng KJ, May A, Kolvraa S, Bohr VA. 2003. Gene expression profiling in Werner syndrome closely resembles that of normal ageing. *Proceedings of the National Academy of Sciences, USA* **100,** 12259–12264.

Levine AJ. 1997. p53, the cellular gatekeeper for growth and division. *Cell* **88,** 323–331.

Lim PO, Woo HR, Nam HG. 2003. Molecular genetics of leaf senescence in Arabidopsis. *Trends in Plant Science* **8,** 272–278.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum* **15,** 473–497.

Nam HG. 1997. The molecular genetic analysis of leaf senescence. *Current Opinion in Biotechnology* **8,** 200–207.

Park SY, Yu JW, Park JS, *et al*. 2007. The senescence-induced staygreen protein regulates chlorophyll degradation. *The Plant Cell* **19,** 1649–1664.

Pletcher SD, Macdonald SJ, Marguerie R, Certa U, Stearns SC, Goldstein DB, Partridge L. 2002. Genome-wide transcript

profiles in ageing and calorically restricted *Drosophila melanogaster*. *Current Biology* **12,** 712–723.

Quirino BF, Noh YS, Himelblau E, Amasino RM. 2000. Molecular aspects of leaf senescence. *Trends in Plant Science* **5,** 278–282.

Rolland F, Moore B, Sheen J. 2002. Sugar sensing and signalling in plants. *The Plant Cell* **14,** Suppl,S185–S205.

Rose MR. 1991. *Evolutionary biology of ageing*. New York: Oxford University Press.

Schippers JHM, Jing HC, Hille J, Dijkwel PP. 2007. Developmental and hormonal control of leaf senescence. In: Gan S, ed. *Senescence processes in plants*. Oxford: Blackwell Publishing, 145–170.

Sgro CM, Partridge L. 1999. A delayed wave of death from reproduction in *Drosophila*. *Science* **286,** 2521–2524.

Sharpless NE, DePinho RA. 2002. p53: Good cop/bad cop. *Cell* **110,** 9–12.

Staswick PE, Su W, Howell SH. 1992. Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. *Proceedings of the National Academy of Science, USA* **89,** 6837–6840.

Stirnberg P, van De Sande K, Leyser HM. 2002. *MAX1* and *MAX2* control shoot lateral branching in Arabidopsis. *Development* **129,** 1131–1141.

Tatar M, Bartke A, Antebi A. 2003. The endocrine regulation of ageing by insulin-like signals. *Science* **299,** 1346–1351.

Thomas H. 2002. Ageing in plants. *Mechanisms of Ageing and Development* **123,** 747–53.

Tyner SD, Venkatachalam S, Choi J, *et al.* 2002. p53 mutant mice that display early ageing-associated phenotypes. *Nature* **415,** 45–53.

Weindruch R, Kayo T, Lee CK, Prolla TA. 2001. Microarray profiling of gene expression in ageing and its alteration by caloric restriction in mice. *Journal of Nutrition* **131,** 918S–923S.

Wilbert SM, Ericsson LH, Gordon MP. 1998. Quantification of jasmonic acid, methyl jasmonate, and salicylic acid in plants by capillary liquid chromatography electrospray tandem mass spectrometry. *Analytical Biochemistry* **257,** 186–194.

Woo HR, Chung KM, Park JH, Oh SA, Ahn T, Hong SH, Jang SK, Nam HG. 2001. ORE9, an F-box protein that regulates leaf senescence in Arabidopsis. *The Plant Cell* **13,** 1779–1790.

Woo HR, Goh CH, Park JH, Teyssendier DIS, Kim JH, Park YI, Nam HG. 2002. Extended leaf longevity in the *ore4-1* mutant of Arabidopsis with a reduced expression of a plastid ribosomal protein gene. *The Plant Journal* **31,** 331–340.

Yoshida S, Ito M, Nishida I, Watanabe A. 2002. Identification of a novel gene *HYS1*/*CPR5* that has a repressive role in the induction of leaf senescence and pathogen-defence responses in *Arabidopsis thaliana*. *The Plant Journal* **29,** 427–437.

Zafon C. 2007. Jekyll and Hyde, the p53 protein, pleiotropics antagonisms and the thrifty aged hypothesis of senescence. *Medical Hypotheses* **68,** 1371–1377.

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

Zou S, Meadows S, Sharp L, Jan LY, Jan YN. 2000. Genome-wide study of ageing and oxidative stress response in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences, USA* **97,** 13726–13731.