Regulation of leaf senescence in Arabidopsis

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Regulation of leaf senescence in *Arabidopsis* -Isolation and characterisation of *onset of leaf death* mutants

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On the cover: Satellite photographs showing the autumn colour changes in the forests around the Great Lakes, USA. The photograph on the front cover was taken on 6th October 2004 at 19:15 UTC, and the one on the back cover on 10th October 2004 at 17:10 UTC. Courtesy of NASA, MODIS Rapid Response at Goddard Space Flight Center (http://rapidfire.sci.gsfc.nasa.gov/).

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Contents

Scope of the thesis

Leaf senescence is the final phase of leaf development. It is visibly marked by leaf colour change, which is associated with active physiological and biochemical processes such as chlorophyll breakdown, RNA and protein degradation, and translocation of nutrients from the senescing leaf to other parts of the plants. It has been well documented that leaf senescence requires *de novo* transcriptional and translational activities and many senescence-associated genes (*SAG*s) have been isolated from a number of plant species including the model plant *Arabidopsis*. However, little is known about the regulation of leaf senescence, or the genes that control *SAG* transcription. This thesis is focused on isolating and characterising senescence regulatory genes in *Arabidopsis*.

In both plants and animals, genes have been identified that can substantially alter lifespan, providing solid evidence to the notion that senescence is subject to genetic regulation. Genes and pathways that are involved in the regulation of leaf senescence were reviewed and compared with those involved in yeast and animal ageing (**Chapter 1**). Senescence is clearly a developmental event and the age of a leaf is the best predictor of when senescence will start. How this developmental feature is related to age-specific gene action was examined. A senescence window concept was proposed to distinguish the differential induction of senescence during development. The experimental approach that could be employed to isolate senescence regulatory genes was discussed.

Leaf senescence is modulated by a variety of plant endogenous hormones. It has been shown that the gaseous phytohormone ethylene regulates the timing of leaf senescence. The relationship between leaf age and ethylene was examined by observing how the effects of exogenously applied ethylene on senescence change with advancing leaf age (**Chapter 2**). The senescence window concept was employed to explain the effects of ethylene on senescence, and was used as the basis for screens for *Arabidopsis* leaf senescence mutants. Several classes of *old* (*onset of leaf death*) mutants were described and it was suggested that ethylene-induced senescence is under complex regulation involving multiple genetic loci. This led to a further study on the interaction between leaf age and ethylene (**Chapter 3**).

To understand the molecular nature of the *OLD* genes and the mechanisms through which they control leaf senescence, cloning of *OLD1* and *OLD3* was carried out and the results are presented in **Chapter 4** and **Chapter 5**. The functions of *OLD1* throughout development and the involvement of hormonal signalling pathways in *old1*-induced phenotypes were dissected. The effects of *OLD1* overexpression were investigated in comparison with those of *old1* mutations. *old3* was shown to encode a mutated isoform of the cytosolic *O*-acetyl-L-serine (thiol) lyase. The effect of the *old3* mutation on sulphur balance, cadmium tolerance and leaf senescence was examined.

In the end of this thesis, the conclusions obtained from the study are highlighted in the **Summary**.

Chapter 1
Ageing in plants: conserved strategies and novel pathways

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Abstract
Ageing increases chaos and entropy and ultimately leads to the death of living organisms. Nevertheless, single gene mutations substantially alter lifespan, revealing that ageing is subject to genetic control. In higher plants, ageing is most obviously manifested by the senescence of leaves, and recent molecular genetic studies, in particular the isolation of *Arabidopsis* mutants with altered leaf senescence, have greatly advanced our understanding of ageing regulation in plants. This paper provides an overview of the identified genes and their respective molecular pathways. Hormones, metabolic flux, reactive oxygen species and protein degradation are prominent strategies employed by plants to control leaf senescence. Plants predominantly use similar ageing-regulating strategies as yeast and animals but have evolved different molecular pathways. The senescence window concept is proposed to describe the age-dependent actions of the regulatory genes. It is concluded that the similarities and differences in ageing between plants and other organisms are deeply rooted in the evolution of ageing and we hope to stimulate discussion and research in the fascinating field of leaf senescence.

Keywords: ageing, leaf senescence, *Arabidopsis*, hormones, metabolic flux, reactive oxygen species, protein degradation

Introduction
Ageing is almost a universal phenomenon in living organisms and in higher plants it is most obviously manifested by the senescence of leaves. Constituting the last part of leaf development, leaf senescence has evolved as an indispensable process to maximise the re-utilisation of nutrients that have been accumulated in the senescing leaves (Leopold, 1961; Bleecker, 1998). Understanding senescence at the molecular level will provide not only information about the regulation of developmental cell death, but also tools to manipulate the senescence process of crops for agricultural development.

Leaf senescence is a genetically regulated developmental programme: sequential events at the morphological, physiological and molecular levels are orchestrated and specific signatures of its stages can be identified. The most prominent symptom of leaf senescence is the visible yellowing, which correlates with physiological and biochemical changes such as dismantling of chloroplasts, drop of chlorophyll content and photosynthetic activities, and degradation of RNA and proteins. Though degenerative in nature, leaf senescence requires active gene expression as envisaged by the identification of so-called senescence-associated genes (*SAG*s) whose expression levels are up-regulated during senescence. To date over 100 *SAG*s have been identified in diverse plant species, and the list of *SAG*s is still increasing. Their expression profiles have been examined during development and under various induction conditions (Smart, 1994; Buchannan-Wollaston, 1997; Gan and Amasino, 1997; Nam, 1997). These exhaustive studies confirm that leaf senescence is a well-defined developmental programme constituting an essential niche in leaf development rather than just being a negative catastrophic process. The identification of such morphological, physiological and molecular events also provide excellent biomarkers for leaf senescence.

Several conclusions can be drawn from studying the developmental aspects of leaf senescence, especially those obtained from analysing the expression profiles of *SAG*s. (1) Leaf senescence is a complex developmental phase involving the actions of many genes from

diverse biochemical pathways. Although the senescence syndrome may look similar phenotypically, the underlying molecular basis could be very different and knock-out of one pathway in the senescence network does not necessarily affect the overall appearance (He et al., 2001). (2) No common *cis*-elements in the promoter regions of the *SAG*s have been recovered suggesting that there are no common regulatory mechanisms controlling *SAG* expression. (3) Many clones of *SAG*s showing up-regulation of their expression have also been found in other biological processes (e.g., pathogenic resistance) indicating that leaf senescence overlaps with other biological processes. Apparently, senescence is modulated by variants at a large array of genetic loci. This complication led many to consider that mutational analyses may not be the best way to study the regulation of leaf senescence (Bleecker and Patterson, 1997; Buchanan-Wollaston, 1997; Quirino et al., 2000).

In contrast, ageing researches outside the plant field show that single gene mutations can substantially alter the lifespan in several organisms ranging from unicellular yeast, to worms, fruit flies and some mammals (Kirkwood and Austad, 2000). This convincingly shows that genetic analysis can indeed be very powerful in dissecting the mechanisms of senescence. Although lagging behind, plant senescence research has made substantial advance in identifying senescence regulatory genes in the past years, thanks to the molecular genetic studies performed with *Arabidopsis* and the completion of its genome sequencing project. Emerging evidence now allows us to have a glimpse of the pathways involved and to compare the molecular strategies between plants and animals.

This review thus aims to provide an overview of the identified genes. They are grouped and described based on the pathways they involve, and comparisons are made with those of the genes in other senescence paradigms in an attempt to find the common features of senescence regulation. The developmental aspect of ageing is discussed in the context of the senescence window. It appears that gene mutations in diverse facets of plant growth and development could alter leaf senescence. A possible explanation for such diversity is discussed from an evolutionary perspective. We do not intend to cover all aspects of senescence but hope to stimulate more discussion and research in the fascinating field of leaf senescence.

Ageing strategies in yeast and animals

Like many other biological processes, our understanding on ageing has been greatly advanced through studies on short-lived laboratory models. Many mutations that change lifespan have been isolated from yeast and animals, and comparative studies showed that insulin signalling, metabolic flux and free radicals are conserved strategies employed to regulate ageing. These findings imply that the genetic control of ageing might have developed in a common ancestor.

The insulin/IGF (insulin growth factor)-1 signalling represents the conserved hormone regulation of lifespan (Finch and Ruvkun, 2001; Kenyon, 2001; Longo and Fabrizio, 2002). Homologous genes in the pathway have been found in ageing paradigms from yeast, to nematode worms, fruit flies and mammals. Interestingly, this pathway also allows animals to sense environmental cues and to adjust growth and development, and controls oxidative stress resistance, food utilisation and reproduction.

The role of metabolic flux in regulating ageing was first shown by caloric restriction, which was first developed from rodent ageing studies and describes a manipulation to reduce the overall energy intake of the animal (30-60% of *ad libitum* intake). Calorie restriction retards the rate of ageing and extends lifespan in a wide spectrum of species (Pugh et al., 1999; Guarente and Kenyon, 2000; Merry, 2002). The effect of calorie restriction has been assumed

to be realised through a global switch in metabolism (Lin et al., 2002; Merry, 2002). It may function through regulating sugar sensing and free radical production. More recently, calorie restriction was shown to share overlap effects with insulin/IGF-1 signalling in regulating lifespan of fruit flies (Clancy et al., 2002).

While increased oxidative damage accelerates ageing, enhanced resistance to oxidative damage can extend lifespan (Finkel and Holbrook, 2000). These findings support the free radical theory of ageing, which states that ageing results from the imbalance between the deterioration resulted from reactive oxygen species (ROS) and the protection by antioxidants, and that the wear and tear on cellular components eventually leads to ageing (Biesalski, 2002).

In addition, ageing in yeast and animals involves epigenetic regulation as envisaged by the transcriptional regulation and mechanisms for maintenance of genomic stability. The SIR2 (silencing information regulator 2) controls transcriptional activities and plays a regulatory role in ageing (Guarente and Kenyon, 2000; Chang and Min, 2002; Roy et al., 2002). Telomere length and telomerase activity are involved in cellular maintenance and their dysfunction caused premature ageing (Young and Smith, 2000; Donehower, 2002). The homologous and non-homologous recombination pathways are involved in the repair of DNA double strand break and mutations in genes in both pathways caused substantial shortening of lifespans (Haber, 2000; Saintigny et al., 2002).

Thus, in yeast and animal ageing paradigms, the insulin/IGF-1 hormone, metabolic flux, free radicals and genome stability play prominent roles in regulating ageing. In the following part, we present molecular genetic evidence to show that regulation of ageing in plants shares similarity to yeast and animals with respect to the strategies employed, but appears to use different molecular pathways.

Hormonal regulation

Plants do not possess an insulin/IGF-1 signalling pathway, but do employ hormonal actions to control lifespan. Actually, a more sophisticated hormonal system has evolved in plants. Up to date, all the identified phytohormones are involved in leaf senescence one way or another. Among the five classic hormones, the roles of ethylene and cytokinin in leaf senescence have long been established. Besides, jasmonic acid, salicylic acid, nitric oxide, and brassinosteroid are also implicated in regulating leaf senescence.

The role of ethylene in leaf senescence is revealed by many studies on ethylene-treated plants and ethylene mutants as well as on transgenic plants (Johnson and Ecker, 1998). Ethylene promotes leaf senescence as demonstrated by the effects of ethylene treatment on advancing visible yellowing and *SAG* induction (Grbic and Bleecker, 1995; Weaver et al., 1998), and by *Arabidopsis* ethylene-insensitive mutants that display delayed leaf senescence (Bleecker et al., 1988; Chao et al., 1997; Oh et al., 1997). However, ethylene is neither necessary nor sufficient for the occurrence of senescence. Senescence eventually occurs in the ethylene insensitive mutants. Thus, ethylene acts to modulate the timing of leaf senescence (Grbic and Bleecker , 1995).

Cytokinins play a master regulatory role in leaf senescence as well. While increasing cytokinin production could delay leaf senescence (Gan and Amasino, 1995; Ori et al., 1999), reducing endogenous cytokinin levels resulted in accelerated senescence (Masferrer et al., 2002). Recently, exciting advances have been achieved in dissecting the components involved in cytokinin signalling (Hutchison and Kieber, 2002; Hwang et al., 2002). Among the genes characterised, only the receptor CKI1 and the response regulator ARR2 appear to be involved

in regulating leaf senescence (Hwang and Sheen, 2001). Further studies are required in order to understand fully the molecular mechanisms of cytokinins' involvement in leaf senescence.

Jasmonates (JAs) were proposed to play a regulatory role in leaf senescence. Early experiments, involving treating leaves or cell cultures with jasmonates, showed that a loss of chlorophyll was induced and the expression of photosynthetic-associated genes was suppressed (reviewed by Creelman and Mullet, 1997). Jasmonates could rapidly induce the expression of chlorophyllase (Tsuchiya et al., 1999) and several *SAG*s (Park et al., 1998; Schenk et al., 2000). In the promoter region of the *OPR1*, two *cis*-elements were found to be required for the up-regulation of *OPR1* by leaf senescence and JA (He and Gan, 2001). He et al. (2002) showed that the endogenous levels of jasmonates increased 4-5 fold during senescence. Yellowing of the detached wild-type leaves after a JA treatment correlated with the induction of *SAG12*, whereas in *coi-1* senescence and *SAG12* expression was not induced at the same conditions. Taken together, these studies indicate that jasmonates have a role in promoting leaf senescence. However, for none of the isolated mutants that are impaired in JA biosynthesis and signalling (Berger, 2001), were aberrant phenotypes in leaf senescence reported, suggesting that jasmonates are not essential for leaf senescence. In addition, transgenic plants that either overexpress allene oxide synthase, jasmonic acid carboxyl methyltransferase, or underexpress lipoxygenase, did not show abnormal leaf senescence. Thus, molecular genetic analysis of jasmonate-related mutants did not generate any crucial link between jasmonate action and leaf senescence, and the role of jasmonates in leaf senescence is still a question of debate.

Physiological analysis has shown that ABA (abscisic acid) could promote leaf senescence, but to date molecular genetic analysis has not generated a crucial link between ABA and leaf senescence (Fedoroff, 2002). Salicylic acid was shown to regulate *SAG* expression and leaf senescence (Morris, 2000). Brassinosteroids could promote leaf senescence and mutants deficient in brassinosteroids showed altered leaf senescence indicating their involvement in leaf senescence (Clouse and Sasse, 1998; Yin et al., 2002). Nevertheless, a systematic study is needed to dissect the regulatory functions of these hormones in leaf senescence.

Metabolic flux

The photoautotrophic nature of plants makes them fundamentally different from animals. Their energy input depends on the available photosynthetic activity, light and CO_2 and altering the available sources could substantially change the process of leaf senescence. Miller et al. (1997) found that elevated CO_2 could accelerate the shift of leaf development from the photosynthetic activity increase phase to the decrease phase. Ludewig and Sonnewald (2000) subsequently showed that this was caused by the earlier onset of leaf senescence. Leaf senescence was also examined in plants with reduced available sources. In *Rubisco* antisense tobacco plants, less dry weight and chlorophyll content was achieved than the wild type at maturity, while the leaf ontogeny was not altered (Miller et al., 2000). The most striking feature of the *Rubisco* antisense plants is that the senescence was markedly prolonged resulting in extended longevity. This pattern is similar to one of the stay-green mutants described in pea (Thomas and Howarth, 2002). More recently, the *Arabidopsis* delayed leaf senescence mutant *ore4-1*, was shown to contain a T-DNA insertion in the plastid ribosomal small subunit protein 17 (*PRPS17)* gene (Woo et al., 2002). The *ore4-1* mutants achieved less dry weight and contained less chlorophyll contents as in the *Rubisco* antisense plants, and more importantly, the photosynthetic system I activity of the *ore4-1* mutants was impaired. These results suggest that disruption of *PRPS17* resulted in reduced chloroplast function and energy input, perhaps

mimicking the effect of calorie restriction in animals. Thus, increased energy input (mimicking overfeeding in animals?) could accelerate leaf senescence, whereas reduced energy input had an opposite effect.

It has been proposed that leaf senescence is initiated when photosynthetic activity drops below a certain threshold level (Hensel et al., 1993). This threshold could be related to leaf sugar levels. Indeed, leaf soluble sugar content increases with leaf age, and growth on media supplemented with sugars could repress photosynthesis associated gene (*PAG)* transcription and translation (Dijkwel et al., 1997; Jang et al., 1997; Wingler et al., 1998). Sugars could specifically inhibit the expression of several *SAG*s associated with dark induction (Fujiki et al., 2001). However, in *SAG12-ipt* (isopentenyl transferase) transgenic tobacco the sugar levels were not different from *SAG12-GUS* plants, although senescence in the former one was substantially delayed (Ludewig and Sonnewald, 2000). In the senescent leaves the soluble sugars showed higher levels than in the non-senescent leaves presumably due to the break-down of chloroplast and cell wall compounds (Quirino et al., 2001). This suggests that increased sugar levels are a consequence rather than a signal to initiate senescence. Exogenous sugars also had different effects on the expression profile of *SAG*s. While enhancing the expression of *SAG21* and *SAG13*, sugars inhibited the expression of *SAG12* (Noh and Amasino, 1999; Xiao et al., 2000). Taken together, the absolute level of sugars appears not to be directly involved in the regulation of leaf senescence. On the other hand, compelling evidence shows that sugar sensing and signalling can influence senescence. In *Arabidopsis* plants overexpressing sense and antisense hexokinase genes (*AtHXK1* and *AtHXK2*), the greening process and the expression profile of *PAG*s and *SAG21* were directly correlated with *AtHXK* expression levels (Jang et al., 1997; Xiao et al., 2000). Similar results were observed in transgenic tomato plants overexpressing *Arabidopsis AtHXK1* (Dai et al., 1999). The *gin2* mutant that has a lesion in the *AtHXK1* gene shows delayed leaf senescence as well as reduced glucose sensitivity (Quirino et al., 2000; Rolland et al., 2002). The *cpr5* mutant that was originally isolated based on the altered pathogen resistance was shown to have sugar hypersensitivity and early leaf senescence (Bowling et al., 1997; Yoshida et al., 2002a).

Thus, altered energy intake or sensing can substantially influence senescence. However, more studies are needed to elucidate the precise molecular mechanisms. It is known that sugars can interact with several distinct signalling pathways such as ABA, ethylene, light and cytokinins, all of which are implicated in regulation of leaf senescence (Smeekens, 2000; Rolland et al., 2002). The effect of sugars on leaf senescence may depend on these interactions.

Free radical theory of ageing

A wealth of data exists on the association between leaf senescence and oxidative damage. During senescence, ROS and oxidative damage increases, whereas the levels of antioxidant enzymes such as SOD, catalase, and ascorbate peroxidase drop (e.g., Jimenez et al., 1998; Ye et al., 2000; Orendi, 2001; Munne-Bosch and Alegre, 2002). Stress-induced senescence is accompanied with an increase in ROS and decrease in antioxidant enzymes (e.g., Hodges and Forney, 2000; Sandalio et al., 2001; Santos et al., 2001). Leaf senescence and the expression of various *SAG*s were promoted in old leaves upon exposure to UV-B or ozone, which are known oxidative damage inducing treatments (Miller et al., 1999; John et al., 2001). In *Arabidopsis* a copper homeostasis gene *CCH* (copper chaperone) was shown to be upregulated by ozone and during leaf senescence (Himelblau et al., 1998) and the expression of a vegetative-storage-protein gene is commonly regulated by copper, senescence and ozone (Mira et al., 2002). An

Arabidopsis cytochrome P450 gene that catalyses oxidative reactions was found to be expressed during leaf senescence (Godiard et al., 1998). These results provided circumstantial evidence that ROS contribute to the progression of leaf senescence. Mutant analysis and studies on transgenic plants provided a more straightforward support for the role of ROS in senescence. Kurepa et al. (1998) reported that the *Arabidopsis* later-flowering mutant *gigantea* was more tolerant to paraquat demonstrating a direct link between the oxidative tolerance and longevity. Alteration in the non-enzymatic antioxidants could influence leaf senescence (Smirnoff, 2001). In addition, transgenic plants, in which the antioxidant enzymes were manipulated, exhibited altered leaf senescence (Orvar and Ellis, 1997; Willekens et al., 1997). Thus the molecular analysis substantiates the direct involvement of ROS in leaf senescence.

ROS have a tight relationship with membrane and lipid dynamics since the membrane-associated NAD(P)H oxidases can sense both endogenous and exogenous stresses and are one of the major generators of ROS (Mittler, 2002). The involvement of lipid metabolism in leaf senescence was demonstrated by studying phospholipid catabolism. In *Arabidopsis* antisense suppression of phopholipase Dα delayed ABA- or ethylene-induced leaf senescence (Fan et al., 1997) and an *Arabidopsis SAG101* gene encoding an acyl hydrolase was shown to be involved in leaf senescence (He and Gan, 2002). Lipids are produced by fatty acid biosynthesis pathways, hence mutations in this pathway were also shown to change leaf senescence (Mou et al., 2000; Wellesen et al., 2001; Mou et al., 2002). Thus, ROS-induced membrane shuffling and lipid metabolism is not a passive wear and tear process but actively involved in leaf senescence.

In summary, there is an intrinsic link between oxidative damage and leaf senescence, and the free radical theory of ageing seems to apply to plant senescence.

Regulation of leaf senescence by protein degradation

Convincing evidence recently demonstrated a link between the plant protein degradation pathways and leaf senescence. Protein degradation can be selective or non-selective. The best-characterised selective protein removal pathway is the ubiquitin-mediated proteolysis pathway via 26S proteasome. The non-selective pathway employs vacuolar proteolysis. Both pathways appear to be involved in leaf senescence as revealed by the isolation of mutants in these pathways. The involvement of ubiquitin-mediated proteolysis in the control of leaf senescence is shown by the recent identification of *ORE9* (Woo et al., 2001) and *DLS1* (Yoshida et al., 2002b). The *ore9* mutation delayed both age-regulated and hormone-induced senescence and *ORE9* was shown to encode an F-box protein (Woo et al., 2001). Interestingly, *ore9/max2* was also recovered in the screen searching for altered shoot lateral branching mutants (Stirnberg et al., 2002). The phenotypes of *ore9* and *max2* mutants resemble those of plants with enhanced cytokinin production or sensitivity, leading to the argument that ORE9 may be involved in ubiquitylating a positive regulator of cytokinin signalling and targeting it for degradation (Frugis and Chua, 2002). The *dls1* mutant showed delayed leaf senescence phenotype and contains a T-DNA insertion in the arginyl-tRNA:protein arginyltransferase (*ATATE1*), which is involved in the N-end rule pathway (Varshavsky et al., 2000), further demonstrating the significance of ubiquitin-mediated protein degradation in the regulation of leaf senescence.

The importance of the non-selective protein degradation via the autophagy pathway in leaf senescence is revealed by two recent reports on the early leaf senescence mutants *apg7* and *apg9-1* (Doelling et al., 2002; Hanaoka et al., 2002). Interestingly, the mRNA and protein

levels of *APG7* and *APG8* in wild type continued to accumulate in senescing wild-type leaves suggesting that the APG8/12 conjugation pathways are up-regulated during senescence. Clearly, the autophagy protein degradation pathway plays an important role in regulating the progression of the senescence syndrome. An intriguing question is why accelerated senescence occurs considering autophagy as a main contributor to the cellular degradation. One possibility may be that the autophagy pathway is responsible for the removal of damaged proteins, and mutations in its components block such function resulting in faster accumulation of damage and early senescence (Grune et al., 1997).

Thus, protein degradation plays an important role in plant senescence regulation. Further research to identify more components in the pathways and their interacting factors will provide additional insight into the molecular mechanisms of leaf senescence.

Genome stability

Senescent tissues are highly stressed and prone to oxidative damage. Yet, leaf cells continue to functionally operate transcriptional and translational activities along the progression of leaf senescence. Therefore, plants must have excellent mechanisms that guard the genome stability until the last moment. In this sense, mutations in maintaining genomic stability and high fidelity transcription activities should cause dramatic changes in senescence as shown in animal paradigms.

Among several identified DNA repair mechanisms in response to oxidative damage, nucleotide excision repair appears to play a pivotal role in ensuring the normal progression of leaf senescence. In *Arabidopsis* several yeast homologous genes that are involved in nucleotide excision repair have been identified through mutational analysis of ultraviolet radiation sensitive phenotypes, namely, *UVH1/RAD1, UVH3/RAD2* and *AtXPB1/RAD25* (Liu et al., 2000; Costa et al., 2001; Liu et al., 2001). UVH1 is homologous to the XPF component of the 5' repair endonuclease, UVH3 to that of XPG, and *AtXPB* encodes a DNA helicase subunit of the core transcription factor IIH complex. All the *Arabidopsis* mutants showed earlier death upon exposure to UV radiation, or ionising oxidation. In the case of *uvh3/rad2*, the early senescence phenotype occurred even in the absence of UV exposure, although the symptoms were less severe. These mutations may cause inhibition or insufficient transcriptional activity that is required for maintaining leaf longevity. This was the case for a mouse mutant containing the mutated *XPD* that showed premature ageing due to the direct defect in transcription (de Boer et al., 2002). These results demonstrate the link between the lack of DNA damage repair and leaf senescence, and that wear and tear on DNA is a common causal factor for ageing, which is conserved between plant and animal kingdoms.

However, clear differences were found as well. One aspect studied was transcriptional regulation by histone deacetylation. In plants three classes of histon deacetylases have been found, but none of them were shown to be specifically involved in leaf senescence (Wu et al., 2000a, b; Lusser et al., 2001; Tian and Chen, 2001; Li et al., 2002). Thus, whether regulation of leaf senescence involves gene silencing by histone deacetylation still needs to prove. Another aspect examined was the dynamics of telomere length and telomerase activity. In *Arabidopsis* several yeast homologue genes that are involved in maintaining telomere length have been identified, and mutations in these genes generated either no abnormal phenotypes in the case of *KU70* (Bundock et al., 2002) and *mim* (Mengiste et al., 1999), or sterile plants in which the senescence phenotypes could only be examined using callus in the case of *rad50* (Gallego and White, 2001). In one extreme situation, a T-DNA knock-out of *Arabidopsis AtTERT* was

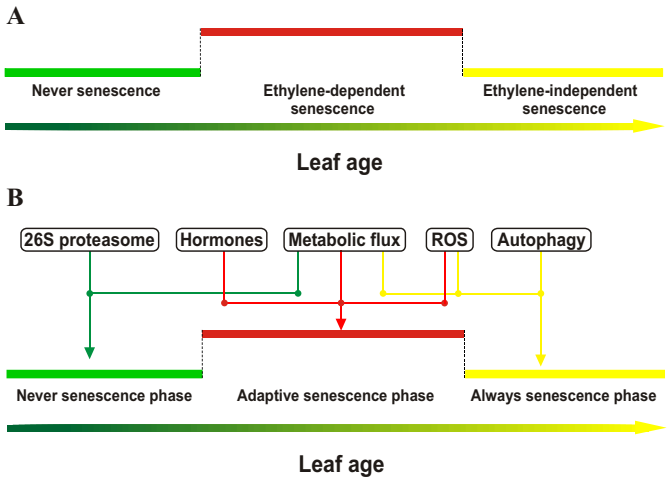

Figure 1. A schematic representation of the senescnece window concept. A. The senescence window as revealed from the effects of ethylene on senescence. B. A tentative model indicating the positions of the major senescence regulators in the senescence window. See text for details.

shown to survive 10 generations without telomerase and plants from the last five generations contained severe cellular damage (Riha et al., 2001). Despite these, the late-generation mutants surprisingly had an extended lifespan of leaves and plants. These results confirmed earlier observations that telomere dynamics is not associated with plant longevity (Riha et al., 1998). This is in striking contrast to animals' response to telomere dysfunction.

The above-mentioned genes are also involved in non-homologous end-joining repair of DNA double-strand breaks, therefore this DNA repair mechanism does not appear to be involved in the regulation of senescence. Double-strand breaks can also be repaired by homologous recombination. In yeast, the *sgs1* mutant contains a mutation in the *RecQ* helicase and showed an increased rate of homologous recombination and premature ageing. Similarly, in human the Werner and Bloom genes are two members of the *RecQ* gene family and patients with a defect in these genes also showed severe premature ageing (Saintigny et al., 2002). Although in plants there are 7 *RecQ-Like* genes (Hartung et al., 2000), whether they are involved in ageing in plants is still not yet clear.

The developmental programme of ageing revealed by the senescence window

Ageing is a developmental programme since the gene expression profiles of ageing organisms are distinctly different from young ones. In plants this is confirmed by the isolation of many genes that are specifically up-regulated before or during leaf senescence. Evolutionary theories also predict that ageing results from the age-specific actions of genes (Kirkwood and Austad, 2000; Partridge, 2001; Hughes et al., 2002; Partridge and Gem, 2002). As discussed below, such age-dependent gene actions can be explained by the senescence window concept.

Figure 1 shows the senescence window concept, which was developed from the studies on the interaction between leaf age and ethylene (Grbic and Bleecker, 1995; Jing et al., 2002). Leaf senescence shows a distinct tri-phase development in relation to the effect of ethylene (Figure 1A). At early leaf growth, ethylene does not induce leaf senescence, and this is the never senescence phase. This phase could be controlled by developmental signals or homeostatic genes such as so-called age-related factors. Only after a defined stage a leaf switches to the second phase, which allows the action of ethylene perceived to promote leaf senescence. This promoting effect operates within a defined time span, marking the ethylene-dependent

senescence phase. In the final phase, senescence proceeds regardless of the absence or presence of ethylene, and this is the ethylene-independent phase. The concept of the senescence window has clear implications. For instance, mutations in genes acting at the three phases, especially those controlling the transition points of the senescence window, may result in predicted senescence phenotypes. This was experimentally confirmed by the isolation and characterisation of ethylene-insensitive mutants and *old* mutants (Grbic and Bleecker, 1995; Jing et al., 2002).

The senescence window concept appears to apply for other plant hormones as well. Cytokinin is an important senescence regulator. Nevertheless, although substantially delayed in leaf senescence, transgenic plants that have extended duration of cytokinin production did senesce eventually (Gan and Amasino, 1995), suggesting that cytokinin action is age dependent. Jasmonates and ABA are traditionally used to induce senescence in detached leaves, but the induction of senescence requires certain duration of priming time. In addition, their effects depend on the age of the incubated leaves. Senescence is induced slowly in young leaves, faster in mature leaves but no further induction occurs in senescent leaves (Weaver et al., 1998). Thus, the common feature is that plant hormones appear to act in a specific age-window to regulate leaf senescence.

The insulin/IGF-1 pathway regulates ageing in an age-dependent manner as well, as shown by a recent elegant study in nematodes (Dillin et al., 2002). When *daf-2* and *daf-16* RNAi treatments were initiated before young adult stage, the lifespans of worms in various treatments showed the same degree of extension, implying that DAF-2 and DAF-16 act to control life-span only when worms reach adulthood. On the other hand, when *daf-2* RNAi was initiated in old wild-type worms, or *daf-16* RNAi was removed from old *daf-2* mutants, the lifespans of treated worms were not altered, suggesting that DAF-2 and DAF-16 show no effects on ageing of worms after certain developmental stages. Similarly, studies in mice and rats also showed that growth hormone only acts in the early stages to regulate lifespan (Bartke et al., 1998; Hauck and Bartke, 2000; Morrissey et al., 2002). Thus, a hormone-regulating senescence window does seem to exist in animal systems.

ROS also have a specific age-window to regulate ageing. In plants, antisense suppression of catalase caused necrosis in old leaves (Willekens et al., 1997). ROS only promoted stress-induced senescence after leaf maturation (Miller et al., 1999; John et al., 2001). Thus, ROS function depends on the developmental stage. In *C. elegans*, increasing oxidative damage by incubating worms at various concentrations of oxygen could substantially reduce the lifespan. However, the drop in the survivorship only occurred 10 days after hatching and this was true for both the wild type and the longevity mutants (Adachi et al., 1998). In WI-38 human fibroblasts, H_2O_2 caused DNA oxidative damage, as a function of age, with less effects on young cells, stronger effects on middle-aged cells and no effects on old cells (Wolf et al., 2002). Taken together, ROS seem to be effective at late developmental stages.

Theoretically, the senescence window concept can be extrapolated to describe the function of any gene involved in senescence. The key feature of the senescence window is that it makes distinctions among the actions of genes. Apparently, genes working at the first phase are the master regulators that integrate the information from various external and internal sources and decide when and how senescence starts. Typical examples of such genes can be those that regulate the homeostasis and those that are essential for survival. The genes working at the second phases presumably are those that govern the duration and speed of senescence. The action of these genes allows some plasticity of the progression of senescence,

making the second phase more prone to modulation for application purposes. Some of such genes are involved in hormonal biosynthesis and signalling. Genes working at the last part of the senescence window may be mainly activated by the second class genes to amplify the effects and start to take actions when the second class genes do not contribute to senescence any more. Nucleases and proteases might be illustrative examples of this class. At this stage, there is no point of return for senescence and cell death is induced.

The aforementioned arguments lead us to propose a tentative model that integrates the major pathways into the senescence window (Figure 1B). The ubiquitin proteolysis pathway is placed at the first phase of the senescence window based on the following evidence: (1) *ore9* and *dls1* mutants showed delayed onset of both natural and hormone-induced leaf senescence, (2) the proteins targeted for degradation by 26S proteasome are often the regulators of hormonal actions (Frugis and Chua, 2002). In contrast, the nature of the autophagy non-selective protein degradation pathway suggests that it might work in the last phase (Grune, 1997). Hormones could be placed at the adaptive senescence phase (see above). ROS might not work in the never senescence phase since adjusting ROS alone was not enough to change the onset of leaf senescence (Creissen et al., 1999; Karpinska et al., 2000). ROS actions could be mediated by plant hormones and MAP kinase signalling (Sharma et al., 1996; Delledonne et al., 2001; Meinhard and Grill, 2001; Orozco-Cardenas et al., 2001; Jonak et al., 2002), or they could also generate direct damage to DNA to induce leaf senescence (e.g., Liu et al., 2001). Thus, we infer that ROS mainly work at the second and the last phase. The effects of changes in metabolic flux are quite broad due to the fact that energy intake could cause global changes in metabolism. On the one hand, the phenotypes of antisense *Rubisco* tobacco plants and *Arabidopsis ore4-1* mutants suggested that metabolic flux could regulate the switch-off of the never senescence phase. On the other hand, sugars could interact with hormones such as ethylene and ABA to adjust the adaptive phase of the senescence window (Rolland et al., 2002). Moreover, calorie restriction could reduce the oxidative damage delaying the occurrence of the always senescence phase (Merry, 2002). Thus, the metabolic flux pathway is proposed to work throughout development. The key feature of this model is that the pathways are placed based on the developmental phases they act on, which to a certain extent is similar to what was proposed to explain ageing in *C. elegans* (Gems, 2000). Although preliminary, the senescence window concept seems to be universal and can be employed to explain the developmental aspects of ageing regulation.

Evolutionary senescence in plants

Evidence presented above illustrates a striking divergence and convergence between plants and animals regarding senescence regulation. At the molecular level distinctly divergent pathways are differentially employed. In animal systems, the insulin/IGF-1 mediated growth and stress response is one of the prominent pathways (Kenyon, 2001). Other prominent mechanisms that dominate the regulation of ageing in animals include the genomic guidance of p53, telomerase and telomere dynamics, DNA damage sensing and repair, and transcriptional activation and inactivation by histone acetylation/deacetylation. These molecular pathways are either not present or do not appear to play an important role in plant ageing. On the other hand, plants have evolved their own unique senescence-regulating mechanisms. These include the modulation of senescence by phytohormones, photosynthetic machinery, and protein degradation. In plants, the chloroplast is thought to be the first origin and target for initiating senescence, whereas in animals the mitochondrion serves as the initiator. This contrasting

divergence may be deeply rooted in the fundamental surviving strategies evolved in plants and animals, one being an autotroph, the other a heterotroph. As a matter of fact, the special features of plant life forms lead to the arguments that most plants do not age as predicted by theories of ageing (Thomas, 2002). However, striking convergence regarding the strategies employed in senescence regulation seems to be present between plants and animals. One important similarity is that senescence is modulated by a diverse array of pathways or a complex network. In addition, all the proven theories of ageing developed from animal paradigms appear to be valid in plants as well with the calorie restriction interventions, free radical theory of ageing and hormonal modulation being the most conspicuous. In this context, there are few differences between plants and animals. Thus, we may infer that plants and animals have evolved conserved strategies for the regulation of senescence, while employing diverse molecular mechanisms that have been shaped during the long history of evolution.

Why senescence involves multiple pathways? What could be the foundation for the divergence and convergence? To answer these questions, we have to ponder the driving force of natural selection that has shaped the life history of life-forms, the evolution of ageing. The evolutionary theory of ageing was developed from the observations made on the survivorship of wild animals. It states that the force of natural selection diminishes with age and has little effect on the actions of genes beyond the life expectancy of a species in its natural environment (Kirkwood and Austad, 2000; Kirkwood, 2002). Several major predictions can be inferred from the evolutionary theory of ageing. (1) Genes do not evolve to solely regulate ageing, rather the genes important for ageing and lifespan are those that control the durability and maintenance of cells. (2) Deleterious mutations will occur in a variety of genes during the late life of organisms that contribute to the senescence phenotypes. (3) Senescence is a genetically-controlled developmental programme, but it has no adaptive advantages. These predictions are apparently applicable for the plant kingdom, except that in plants leaf senescence is a recruited nutrient recycle programme and hence is considered to have a strong adaptive advantage (Bleecker, 1998). Clearly, the evolutionary basis of senescence is in agreement with the presence of multiple pathways and explains why plants and animals share similar and divergent strategies.

Concluding remarks

A complex network consisting of multiple pathways controls senescence. In order to understand the whole scenario, dissecting the individual pathways is crucial. To date, protein degradation, hormonal modulation, metabolic flux, and ROS appear to be the prominent pathways. Although several components in these pathways have been identified, a lot more efforts are needed to clearly illustrate the precise molecular mechanisms. In addition, the interactions among them should be pursued.

Currently, we are at an exciting time, when most of the technologies required to answer the senescence questions are in place. A concerted effort coupled with multiple approaches should unravel the molecular mechanisms of senescence. Particularly important is the mutational analysis approach. A major advantage of this widely proven approach is that it does not require any prior knowledge of how senescence occurs or what kinds of genes are involved. Most genes and pathways involved in senescence have been identified through mutational analysis. This, in complementation with genome-wide approaches, will help to build a complete picture of the regulation of senescence in plants.

Chapter 2
***Arabidopsis onset of leaf death* mutants identify a regulatory pathway controlling leaf senescence**

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Abstract
The onset of leaf senescence is controlled by leaf age and ethylene can promote leaf senescence within a specific age window. We exploited the interaction between leaf age and ethylene and isolated mutants with altered leaf senescence that are named as *onset of leaf death* (*old*) mutants. Early leaf senescence mutants representing three genetic loci were selected and their senescence syndromes characterised using phenotypical, physiological and molecular markers. *old1* is represented by three recessive alleles and displayed earlier senescence both in air and upon ethylene exposure. The etiolated *old1* seedlings exhibited a hypersensitive triple response. *old2* is a dominant trait and the mutant plants were indistinguishable from the wild-type when grown in air but showed an earlier senescence syndrome upon ethylene treatment. *old3* is a semi-dominant trait and its earlier onset of senescence is independent of ethylene treatment. Analyses of the chlorophyll degradation, ion leakage and *SAG* expression showed that leaf senescence was advanced in ethylene-treated *old2* plants and in both air-grown and ethylene-treated *old1* and *old3* plants. Epistatic analysis indicated that *OLD1* might act downstream of *OLD2* and upstream of *OLD3* and mediate the interaction between leaf age and ethylene. A genetic model was proposed that links the three *OLD* genes and ethylene into a regulatory pathway controlling the onset of leaf senescence.

Key words: *Arabidopsis,* ageing, ethylene, leaf senescence, *old* mutants, senescence-associated genes

Introduction
Leaf senescence is the final phase of leaf development and in nature it is evident by the golden autumn colours. During senescence a salvage process is activated by which nutrients are mobilised from the senescing leaf to other parts of the plant, such as young leaves and/or developing seeds (Nooden, 1988). For this, leaf cells undergo orchestrated changes in morphology, structure and metabolism, which is referred to as the senescence syndrome (for a review, see Bleecker and Patterson, 1997). Leaf senescence is visibly marked by the colour change from green to yellow and red, which results both from the preferential degradation of chlorophylls compared to carotenoids, and from the concomitant synthesis of anthocyanins and phenolics (reviewed by Matile et al., 1999). In *Arabidopsis thaliana*, the visible yellowing and the chlorophyll loss are widely used to stage the progression of senescence, which reproducibly correlate with other biochemical changes that occur during leaf senescence (Hensel et al., 1993; Lohman et al., 1994).

The execution and completion of senescence requires *de novo* transcriptional and translational activities (for a review see Nooden, 1988). Genes whose transcriptional levels are specifically up-regulated in senescing leaves have been cloned and are collectively termed as senescence-associated genes (*SAG*s) (reviewed by Buchanan-Wallaston, 1997; Nam, 1997). In *Arabidopsis* there are dozens of *SAG*s and their expression patterns have been studied in age-regulated senescence and under various induction conditions. These *SAG*s serve as informative molecular markers for monitoring the progression of senescence. However, genes that define the onset of senescence and those that regulate the expression of these *SAG*s, have not been well characterised yet.

Various factors influence the onset of leaf senescence. Leaf senescence is an intrinsic age-dependent process, and its onset appears to be regulated by the age of individual leaves (Hensel et al., 1993). Senescence is also prematurely induced by a range of external factors such as darkness, detachment, drought and pathogen attack (Park et al., 1998; Weaver et al., 1998; Pontier et al., 1999; Quirino et al., 1999). Under these induction conditions distinct sets of *SAG*s are induced, suggesting the existence of a complex regulatory network. In addition, plant hormones have been implicated in regulating leaf senescence (Gan and Amasino, 1995; Morris et al., 2000; He et al., 2002).

The role of the volatile phytohormone ethylene in leaf senescence is revealed by studies on ethylene-treated plants and ethylene mutants as well as on transgenic plants. On the one hand, ethylene promotes leaf senescence. In wild-type *Arabidopsis* plants, ethylene treatment can advance the visible yellowing and *SAG* induction in leaves that are primed to senesce (Grbic and Bleecker, 1995; Weaver et al., 1998). *Arabidopsis* ethylene-insensitive mutants, exemplified by *etr1*, *ein2* and *ein3*, display delayed leaf senescence (Bleecker et al., 1988; Grbic and Bleecker, 1995; Chao et al., 1997; Oh et al., 1997; Hua and Meyerowitz, 1998). On the other hand, ethylene is neither necessary nor sufficient for the occurrence of senescence. Senescence eventually occurs in the ethylene insensitive mutants. Ethylene constitutive response and overproduction mutants such as *ctr* and *eto* do not show premature leaf senescence as could be expected (Guzmàn and Ecker, 1990; Kieber et al., 1993). In tomato plants carrying *Never ripe* mutation or with the antisense suppression of the ACC oxidase, the progress of leaf senescence is not retarded once senescence has started (Lanahan et al., 1994; John et al., 1995). Together, these studies suggest that ethylene does not directly regulate the onset of leaf senescence. It acts to modulate the timing of leaf senescence.

Our research aims to identify genes that control the onset of leaf senescence. Such genes can be approached by isolating mutants that show altered senescence phenotypes. Oh et al. (1997) isolated *ore* mutants with delayed leaf senescence by screening dark-incubated detached leaves. Among the mutants isolated 2 were allelic to *ein2*, further indicating the importance of ethylene's involvement in leaf senescence. *ORE9* has been cloned and shown to encode an F-box protein suggesting the involvement of the ubiquitin-mediated degradation of proteins in the regulation of leaf senescence (Woo et al., 2001). Using a similar dark screen approach, Yoshida et al. (2002a) isolated the *hys1* mutant that shows early leaf senescence phenotypes. *hys1* turned out to be allelic to the *cpr5* mutants, which were initially isolated in screens for altered pathogen-defence responses (Bowling et al., 1997) and for trichome mutants (Kirik et al., 2001).

As discussed above, the nature of ethylene's involvement in leaf senescence makes it an excellent means for the isolation of senescence mutants. We exploited the responses of *Arabidopsis* leaves to ethylene and found that ethylene promotes senescence within a specific age window. By utilising this knowledge, we have isolated mutants that are collectively named as *onset of leaf death* (*old*) mutants and describe here the characterisation of mutants with early leaf senescence phenotypes. The phenotypic, physiological, and molecular genetic characteristics of the mutants show that we have identified novel mutations. The importance and the positions of the *OLD* genes in the senescence-regulating network are discussed, and a genetic model that links the control of leaf senescence by *OLD* genes and the action of ethylene is proposed.

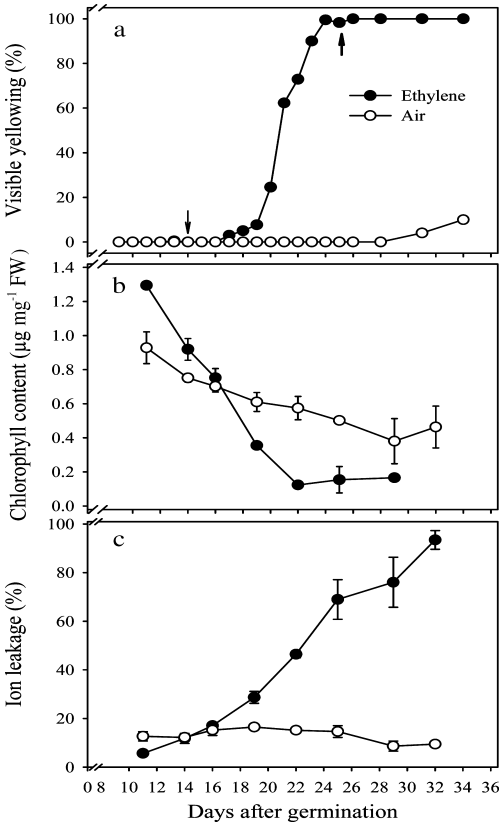

Figure 1. The effect of ethylene on visible yellowing, chlorophyll content and ion leakage of *Arabidopsis* cotyledons.
L*er*-0 plants were grown either continuously in air for the indicated days, or first in air until 3 d before the indicated days and then exposed to 10µll^{-1} ethylene. Cotyledon yellowing (**a**) was subsequently scored as the percentage of yellow cotyledons versus the total number of cotyledons and chlorophyll contents (**b**) and ion leakage (**c**) quantified. Cotyledons with over 10% yellow area of the blade were judged as yellow (corresponding to S1 described by Lohman et al., 1994). The visible yellowing was observed on at least 100 plants for each data point and the results for chlorophyll content and ion leakage were shown as mean±sd of three replicates. Arrows in (**a**) indicate the time points when the screen for early or late leaf senescence mutants was performed at the end of the 3-d ethylene exposure. Note that the initial drop of chlorophyll content in (**b**) coincided with the expanding cotyledons until 16 days.

Results

Ethylene treatment of mutagenised *Arabidopsis* plants allows for the isolation of leaf senescence mutants

As a first step towards establishing a mutant screen system, we tested the effect of a 3-d ethylene treatment on the progression of leaf senescence. Figure 1 shows the effect of ethylene treatment on the visible yellowing, chlorophyll content and ion leakage of *Arabidopsis* cotyledons. In air-grown plants visible yellowing appeared on cotyledons from 30 d onwards (Figure 1a), and all the plants had yellow cotyledons after 45 d (data not shown). Upon ethylene treatment, visible yellowing was not observed in plants younger than 17 d. Thereafter visible yellowing was induced in cotyledons and the cotyledons became yellow in all the plants older than 24 d. Thus exogenous ethylene does promote the cotyledon yellowing, but its effect depends on the age of cotyledons. Similar trends of visible yellowing were sequentially observed in the rosette leaves. For example, in ethylene-treated first rosette leaves yellowing started from 24 d and all the first rosette leaves became yellow after 28 d, while in the ethylene-treated second rosette leaves yellowing started at 25 d and they all became yellow after 29 d (data not shown). These results showed that ethylene induces visible yellowing of *Arabidopsis* cotyledons and leaves in an age-dependent manner.

Table 1. Genetic segregation analysis of *old* mutants

Male	Female	Generation	Wild-type	Mutant	χ^2	P
old1-1	L*er*-0	F_1	17	0		
		F_2	599	217	1.105	>0.29
old1-1	*old1-2*	F_1	0	29		
old1-1	*old1-3*	F_1	0	22		
old2	L*er*-0	F_1[a]	0	17		
		F_2	97	237	2.910	>0.08
old2	Col-0	F_1	54	0		
		F_2	168	190	1.469[b]	>0.22
old3[c]	L*er*-0	F_1	6	7		
		F_2	245	482/231[d]	0.447[e]	>0.79
old3[c]	Col-0	F_1	140	0		
		F_2	355	128/30[d]	0.136[f]	>0.93

[a]The *old2* heterozygotes had phenotypes similar to that of the parental *old2* homozygote. The phenotype scoring was carried out on 24-d-old plants treated with 10 µl l^{-1} ethylene for 3 d. The criteria were: wild-type plants with 0-3 yellow cotyledons and/or leaves, *old2* plants with 2 yellow cotyledons and at least 2 yellow leaves.

[b]A 7:9 ratio of wild-type:*old2* was tested.

[c]The pollen from *old3* heterozygotes was used for pollination since the *old3* homozygotes were lethal. Two types of F_1 plants were obtained in the back-crosses to Ler-0, whereas in the back-crosses to Col-0 all the F_1 progeny showed the wild-type phenotypes.

[d]The progeny of the *OLD3/old3* F_1 plants were used for segregation test. These numbers represent heterozygous/homozygous *old3*.

[e]A 1:2:1 ratio of Wild-type:intermediate:mutant was tested.

[f]A 11:4:1ratio of wild-type:intermediate:mutant was tested.

To further validate whether the effect of exogenous ethylene in inducing senescence was a function of leaf age, we followed changes in chlorophyll content and ion leakage. As shown in Figure 1b and 1c, a drop in the chlorophyll content and an increase in the ion leakage were associated with the progression of the visible yellowing, suggesting a well-defined correlation between the visible symptoms and the physiological changes. Interestingly, a vast increase in the ion leakage occurred after the chlorophyll content dropped to the minimal, showing that the occurrence of the cellular membrane damage lags behind the chlorophyll degradation. Similar results were reported by Woo et al. (2001). Taken together, these results showed that there is an intrinsic linkage among visible yellowing, chlorophyll degradation, and cellular membrane damage in ethylene-induced senescence and confirmed that the effect of ethylene on leaf senescence relies on leaf age (Grbic and Bleecker, 1995; Weaver et al. 1998).

The above experiments provided evidence for the following notions. (1) *Arabidopsis* cotyledons have to reach a defined developmental stage to perceive ethylene's action in inducing senescence. (2) An ethylene-treated mutagenised plant that shows yellow cotyledons before 17 d or no yellow symptoms after 24 d, can be considered a putative early or late leaf senescence mutant, respectively. These notions led to the establishment of a screen system for isolating leaf senescence mutants. Both early and late leaf senescence mutants were isolated as described in Experimental procedures and are names as *old* (*onset of leaf death*) mutants. In this paper, we focus on early leaf senescence mutants and report the

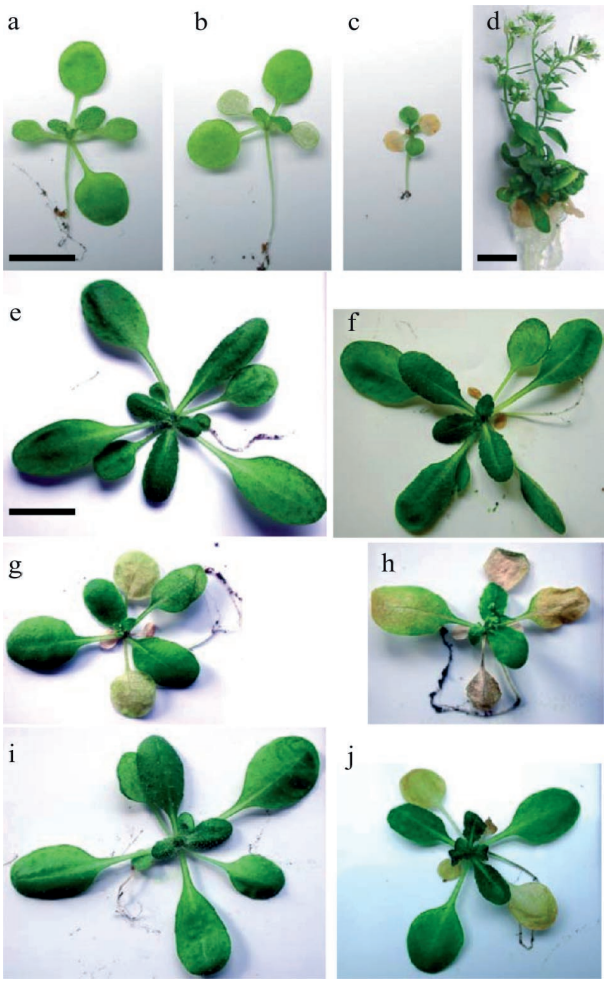

Figure 2. Phenotypes of old mutants in comparison with the wild-type.
(a-c) 16-d air-grown L*er*-0, *old1-1*, and *old3*.
(d) 50-d *old*3 grown in Murashige and Skoog medium plus 1% sucrose.
(e-f) 24-d L*er*-0 grown in air or with 3-d ethylene treatment.
(g-h) 24-d *old1-1* grown in air or with 3-d ethylene treatment.
(i-j) 24-d *old2* grown in air or with 3-d ethylene treatment.
The bar in a represents 0.5 cm and those in d and e represent 1 cm. Pictures a to c and e to j were taken at the same magnification.

characterisation of mutations in 3 genetic loci that are implicated in the regulation of leaf senescence.

Early leaf senescence mutants fall into 3 complementation groups

The early senescence mutants were sub-grouped into three classes, depending on changes in their phenotypes before and after ethylene treatment in comparison with the wild-type. Class I mutants show early senescence symptoms in air, and the symptoms are further enhanced by ethylene treatment. Class II mutants do not have early senescence phenotypes in air, but they exhibit accelerated senescence upon ethylene exposure. Class III mutants senesce earlier than wild-type in air, but ethylene treatment does not induce senescence in additional leaves. We selected representative mutants of each class from the L*er*-0 accession for further study. Back-cross and complementation tests placed them into three loci: *old1*, *old2* and *old3* (Table 1).

The *old1* locus is represented by 3 recessive alleles isolated from independent M_2 pools. We selected *old1-1* for future studies. The *old1-1* plants have a strong early leaf senescence phenotype. After ~14 d of growth, *old1-1* cotyledons became yellow, while wild-type cotyledons were still green (Figures 2a and 2b). By 24 d yellowing was observed in the first 2 rosette leaves as well, and ethylene treatment strongly promoted leaf senescence in *old1-1* plants (Figures 2g and 2h). The adult *old1-1* plants start bolting and flowering earlier (bolts arose in *old1-1* with 4-6 rosette leaves and in L*er*-0 with 8-9 rosette leaves) and had a reduced size and less progeny. The *old1-1* mutation was mapped to the bottom of chromosome 5, ~3 centiMorgans south of the single nucleotide polymorphism (SNP) marker SGCSNP84.

The *old2* plants are indistinguishable from wild-type plants in air (Figures 2e and 2i), but ethylene triggers accelerated senescence. After ethylene treatment at 21-24 d, *old2* plants showed yellow cotyledons and first 2 rosette leaves, whilst wild-type plants had yellow cotyledons (Figures 2f and 2j). Genetic analysis revealed that *old2* is an ethylene-required dominant trait and that there is a dominant suppressor of *old2* in Col-0 (Table 1). These characteristics make mapping a rather time-consuming process. The *old2* mutant belongs to Class II early leaf senescence mutants, and among 12 mutants isolated 6 showed similar phenotypes and complex genetic behaviour, including the presence of a dominant suppressor in Col-0. It seems likely that they represent the same locus, but this needs to be confirmed by further genetic analysis.

One single *old3* allele was identified. When grown in soil, the homozygous *old3* mutant is rosette-lethal and plants stop growing when the second pair of rosette leaves appears (Figure 2c). Interestingly, *old3* can be rescued by culture on medium with 1% sucrose, although the plants still senesce earlier (Figure 2d). Ethylene treatment enhanced the speed of senescence in cotyledons but did not induce yellowing in additional leaves. A heterozygous plant with intermediate *old3* phenotypes was back-crossed to the parental line L*er*-0, and the progeny tests showed that *old3* is a semi-dominant allele (Table 1). In all the F_1 plants of an *old3* heterozygote by Col-0 cross, the wild-type phenotype was observed indicating that a suppressor exists in Col-0. Subsequent observations of 39 independent F_2 populations showed that 20 of them contained the *old3* mutation and the segregation ratio manifested a duplication of a semi-dominant gene in Col-0. Plants homozygous for both the suppressor and the *old3* loci were used for mapping, and the two loci were placed at the lower arm of chromosome 3, ~4 centiMorgans south of simple sequence length polymorphic (SSLP) marker K11J14, and at the upper arm of chromosome 4, ~3.6 centiMorgans north of CAPS marker G4539a.

Thus, mutants with diverse early leaf senescence phenotypes were isolated, and genetic analysis indicated the presence of at least 3 genetic loci that are involved in the regulation of leaf senescence.

The senescence syndrome is advanced in *old* mutants

We set up and performed mutant screen based on the senescence phenotypes of cotyledons due to the advantage that the screen could be done in a large scale within limited space and a relatively short time. Initial analyses of the three mutants indicated that the earlier senescence also sequentially occurred in rosette leaves. Thus, we carried out further physiological and molecular characterisation of the senescence syndrome using cotyledons, specific rosette leaves and the whole rosette, respectively.

To examine the physiological changes during senescence, the chlorophyll content and the ion leakage were measured in whole rosette, cotyledons and different rosette leaves from 11-

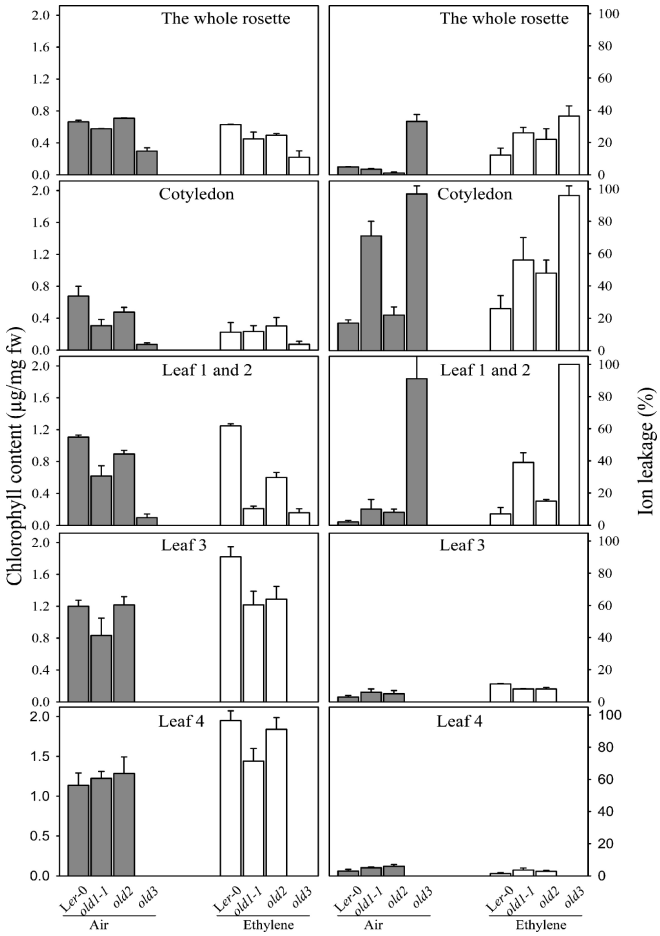

Figure 3. Comparison of chlorophyll content and ion leakage between the wild type and the three *old* mutants. L*er*-0 plants and *old* mutants were grown either in air for 24 d, or first in air until 21 d and then exposed to 10 µl l^{-1} ethylene for 3 d, and the whole rosette or leaves at specific positions were isolated and the chlorophyll content and the ion leakage quantified. Results are shown as mean±sd of at least three replicates. Note the chlorophyll content results between ethylene treatment and air were not comparable due to the inhibitory effect of ethylene on cell expansion.

d, 16-d, 24-d, 30-d and 40-d-old plants. Results for the 24-d samples are presented in Figure 3. In the whole rosette samples isolated from air-grown seedlings, a lower chlorophyll content was found in *old1* and *old3*, while a higher ion leakage was only observed in *old3*, but not in the *old1-1* samples. No differences were found between *old2* and the wild-type. Upon ethylene treatment, samples of the *old* plants showed lower chlorophyll content and higher ion leakage compared to the wild-type. These results were consistent with the visible yellowing of the mutants and the effect of ethylene in inducing visible yellowing (Figure 2), suggesting an overall acceleration of leaf senescence in the *old* mutants.

More detailed analyses using cotyledons, the rosette leaves 1/2, 3 and 4 also showed the advanced leaf senescence in the *old* mutants. For air-grown samples, the chlorophyll content and ion leakage of *old1-1* and *old3* were significantly different from those of *old2* and the wild-type, except the rosette leaf 4 from *old1-1*. Upon ethylene treatment, the chlorophyll content of cotyledons dropped to the minimal for all the plants and no differences were observed, but the ion leakage of the *old* mutants was still higher than that of wild-types. In ethylene-treated rosette leaves 1/2 large differences were observed between *old* mutants and

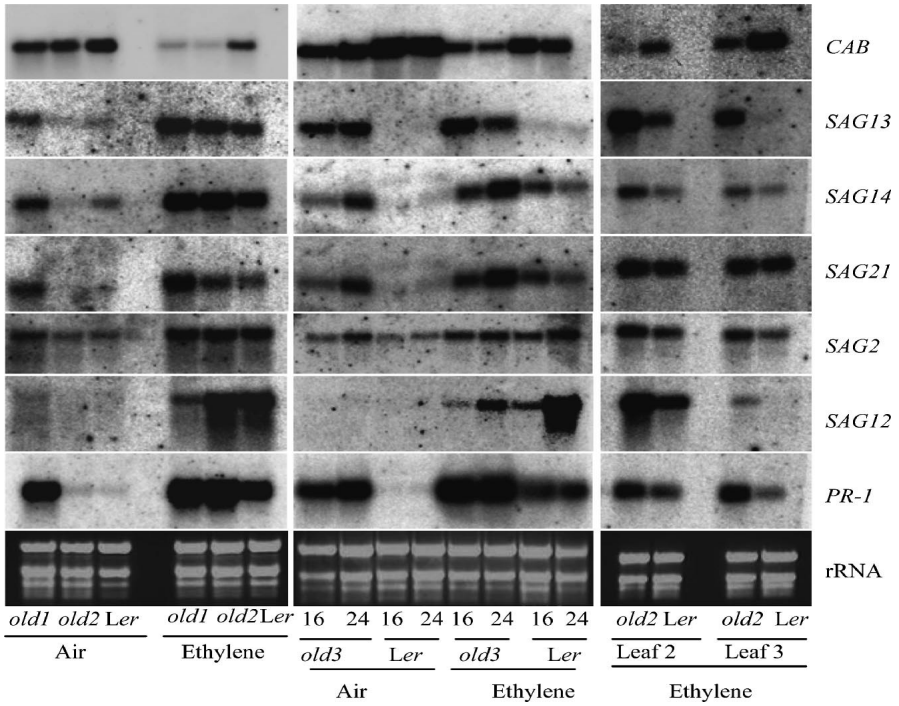

Figure 4. Northern hybridisation analysis showing the abundance of the mRNA of various genes in *old* mutants and wild-type plants.

The left column shows RNA samples from the whole rosette of 24-d *old1-1*, *old2* and the wild-type (L*er*-0) plants. The middle column shows RNA samples from the whole rosette of 16-d and 24-d *old3* and the wild-type plants. The right column shows RNA samples from the rosette leaf number 2 and the green leaf number 3 of the 24-d *old2* and wild-type plants with a 3-d ethylene treatment. For samples in the left and the middle columns 10 µg total RNA was loaded per lane, and for samples in the right column 5 µg RNA was loaded. The sources for each gene are shown in Table 2.

Table 2. Genes used fro hybridisation experiments

Gene names	Encoded protein	References
CAB	Chlorophyll a/b binding protein	Leutwiler et al., 1986
PR-1	Acidic pathogen-related protein	Uknes et al., 1992
SAG2	Cysteine protease	Hensel et al., 1993
SAG12	Cysteine protease	Lohman et al., 1994
SAG13	Short chain alcohol dehydrogenase	Lohman et al., 1994
SAG14	Blue copper-binding protein	Lohman et al., 1994; Weaver et al., 1998
SAG21	Late embryogenesis associated protein	Weaver et al., 1998

the wild-type for both parameters, but in rosette leaves 3 and 4 the *old* mutants differed from the wild-type in the chlorophyll content, not in the ion leakage, further showing the sequential occurrence of chlorophyll degradation and membrane damage. Together, lower chlorophyll contents and higher amounts of ion leakage were observed in air-grown *old1-1* and *old3* and in all ethylene-treated *old* samples, and the younger the leaf, the less the differences between

the *old* mutants and the wild-type, indicating that leaf senescence in the *old* mutants occurred earlier and was controlled by leaf age. These results also suggested that although the mutants were isolated on the basis of the altered senescence phenotypes of cotyledons, the senescence of the rosette leaves was similarly affected.

We subsequently performed a detailed molecular characterisation by Northern blot analysis of the mRNA abundance of the genes listed in Table 2. Changes in the mRNA levels of the tested genes fell into distinct classes (Figure 4). The first class is represented by *CAB* whose mRNA levels reduce during senescence. The levels of *CAB* mRNA were already lower in air-grown *old* plants and more strongly reduced upon ethylene treatment. The second class includes *SAG13*, *SAG14* and *SAG21*. In air, the mRNA levels of these genes in *old1* and *old3* were higher than those in *old2* and the wild-type. Upon ethylene treatment, more pronounced increases in the mRNA levels of these *SAG*s were observed in the *old* mutants in comparison with the wild-type. The third class is shown by *SAG2*, which showed a basal mRNA level that was higher in air-grown *old1* and *old3* plants, consistent with the early occurrence of senescence in these two *old* mutants. Upon ethylene treatment, the *SAG2* mRNA levels did not appear to be higher in *old* mutants, although the mutants showed stronger senescence. The mRNA level of *SAG2* is also increased by exogenous ethylene in green leaves besides being up-regulated during senescence (Hensel et al., 1993; Grbic and Bleecker, 1995), which might be the reason why in ethylene-treated samples little difference was detected between *old* mutants and the wild-type plants.

Changes in the *SAG12* mRNA levels distinctly differed from all the other tested *SAG*s and formed one class on its own. Like in the wild-type, air-grown *old2* plants showed no detectable level of *SAG12* mRNA as expected, but the *SAG12* mRNA levels were very low in air-grown *old1* and undetectable in *old3*, both having prominent senescence symptoms. Upon ethylene treatment, *old2* appeared to have a slightly higher level of *SAG12* mRNA than the wild-type. However, *old1* and *old3* showed lower levels of *SAG12* mRNA than the wild-type, which was again unexpected since much stronger senescence symptoms were evident in these two *old* plants.

Genes conferring resistance to pathogen attack are also highly expressed during senescence (Quirino et al., 1999; Morris et al., 2000). Thus we analysed the expression profile of *PR-1* in *old* mutants. Changes in the *PR-1* mRNA levels showed similar patterns to those of *SAG13*, *SAG14* and *SAG21*, high in air-grown *old1* and *old3* but similarly low in air-grown *old2* and the wild-type. Compared to the wild-type, the mRNA levels increased to much higher levels in ethylene-treated *old* mutants. Therefore, *PR-1* expression was enhanced in the *old* mutants.

The particular phenotype of *old2* mutants prompted us to perform more detailed gene expression analysis on ethylene-treated leaves. The 21-d *old2* and wild-type plants were exposed to ethylene for 3 d, and the *CAB* and *SAG* mRNA levels from the second and third rosette leaves analysed. In this experiment, over 95% of the second rosette leaves were still green in the wild-type after ethylene treatment, whereas over 90% showed signs of senescence in the *old2* plants. For the third rosette leaves only the green ones were used for both *old2* and the wild-type, although ~50% of the *old2* third rosette leaves were yellow. Compared to the wild-type controls, *old2* leaves had lower levels of *CAB* mRNA and higher mRNA levels of the tested *SAG*s. Crucially, even in the green third rosette leaves, the mRNA levels of the tested genes showed large differences between *old2* and the wild-type. In particular, the mRNA levels of *SAG12* and *SAG13*, which are believed to be good markers for natural leaf

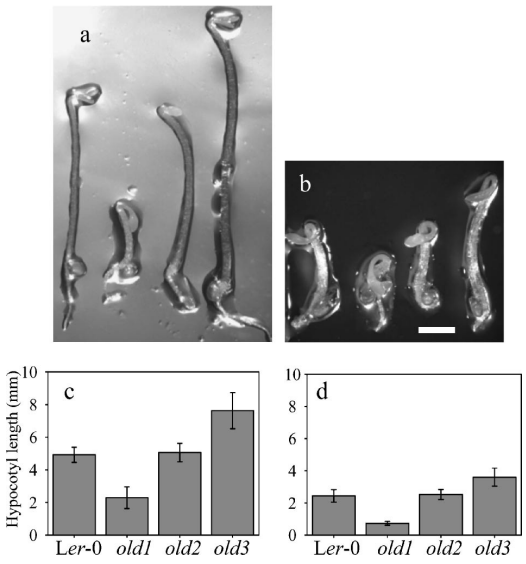

Figure 5. The triple response in the wild-type (L*er*-0) and *old* mutants.
(a) Wild-type and *old* seedlings in the absence of ACC.
(b) Wild-type and *old* seedlings in the presence of 10μM ACC.
(c) The hypocotyl length of wild-type and *old* seedlings in the absence of ACC.
(d) The hypocotyl length of wild-type and *old* seedlings in the presence of 10μM ACC. Seeds were surface sterilised with 25% bleach, plated on to Murashige and Skoog media with and without 10 μM ACC, and grown in darkness at 21°C for 5 d. The hypocotyl length was measured using at least 50 seedlings per treatment and shown as mean±sd.

senescence (Weaver et al., 1998), were prominent in *old2* but undetectable in the wild-type. This comparison more clearly demonstrated the advancement of leaf senescence in *old2*.

old mutants exhibit different ethylene responses

The progression of leaf senescence in the *old* mutants exhibited distinct patterns before and after ethylene treatment, thus the ethylene-signalling pathway was further studied in these *old* mutants by the triple response bioassay (Figure 5). The etiolated *old* mutants already displayed differences in hypocotyl length even in the absence of ACC (Figure 5a and 5c). *old1-1* seedlings showed a shorter hypocotyl. The hypocotyl of *old3* was longer than that of the wild-type, while there was no significant difference between *old2* and the wild-type. When 10 μM ACC was added, *old1-1* displayed an extremely exaggerated triple response, the whole seedling formed a ring-like shape (Figure 5b and 5d). At this ACC concentration, *old3* persistently showed a longer hypocotyl, whilst the hypocotyl of *old2* was not different from that of wild-type. These results suggest that *old1-1* may be hypersensitive to ethylene, *old3* responds to ethylene but sustains a longer hypocotyl, and the triple response is not altered in *old2*.

The *old* mutants may work in a common regulatory pathway

Since the three *old* mutants showed early senescence phenotypes and similar *SAG* expression profiles, they may work in a common regulatory pathway controlling leaf senescence. We performed double mutant analysis to determine the potential genetic interactions between the *OLD* genes. As shown in Table3, when *old1-1* was crossed to *old2*, the plants with *old1-1* phenotypes segregated in a 1:3 ratio; while in the F_2 plants of an *old1-1* by *old3* cross plants showing *old1-1* and *old3* phenotypes segregated in a 3:4 ratio, which suggests that *OLD1* might work upstream of *OLD3* and downstream of *OLD2*. Like *old3* the *old1-1/old3* double mutant is also rosette-lethal. The results from testing relationship between *old2* and *old3*

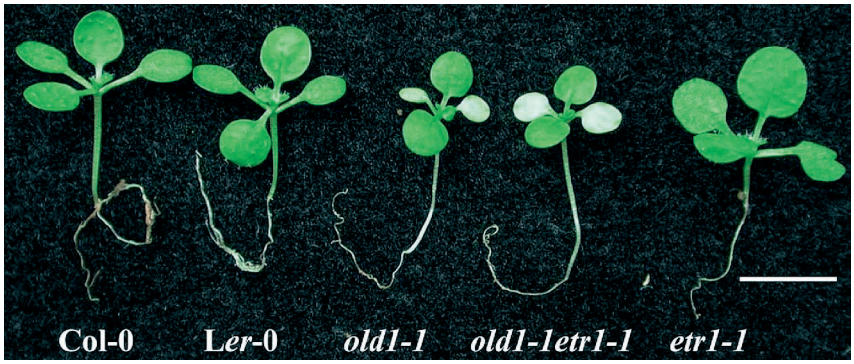

Figure 6. Phenotypes of *old1-1*, *etr1-1* and *old1-1etr1-1* mutants.
Seedlings were grown in conditions described in Experimental procedures for 17 d and photographed. Bar represents 1 cm.

Table 3. Segregation analysis of F_2 progeny of crosses among three *old* mutants

Crosses	WT	*old1-1*	*old2*	He-*old3*	H-*old3*	χ^2	P
old1-1xold2	51	87	163	-	-	2.59[a]	>0.27
old1-1xold3	75	102[b]	-	203	124	5.69[c]	>0.12
old2xold3	13	-	48	148	80	3.04[d]	>0.38

Phenotype scoring was detailed in Experimental procedures. WT: wild type; He: heterozygous; H: homozygous
a: A 3:4:9 ratio of WT:*old1-1*:*old2* was tested.
b: *old1-1old1-1/OLD3old3* and *old1-1old1-1/OLD3OLD3* showed *old1-1* phenotypes.
c: A 3:3:6:4 ratio of WT:*old1-1*:He-*old3*:H-*old3* was tested.
d: A 1:3:8:4 ratio of WT:*old2*:He-*old3*:H-*old3* was tested.

were also consistent with putting *OLD3* downstream of *OLD2*. Overall, results of the epistatic analysis correlated well with the phenotypes of the mutants and the results from *SAG* expression analysis, implying that the *OLD* genes may control a common regulatory pathway for leaf senescence.

Owing to the particular phenotypes of *old1-1*, we considered that *OLD1* may act at the linking point between ethylene- and age-regulated leaf senescence and constructed the double mutant of *old1-1* and *etr1-1*. As shown in Figure 6, at 17 d the air grown *old1-1etr1-1* showed senescing cotyledons as in the *old1-1*, suggesting that the earlier onset of leaf senescence in *old1-1* is independent of ethylene perception. On the other hand, when treated with ethylene, the double mutant failed to show a more exaggerated senescence syndrome as in the *old1-1* indicating that the progression of leaf senescence in *old1-1* does require a functional ethylene pathway. Taken together, these results suggest that *OLD1* mediates the actions of ethylene and age-related factors in the regulation of leaf senescence.

Discussion

The senescence window

In an attempt to identify genetic loci that define the onset of leaf senescence, the interaction between ethylene and leaf age was studied in *Arabidopsis*. The effect of ethylene in inducing

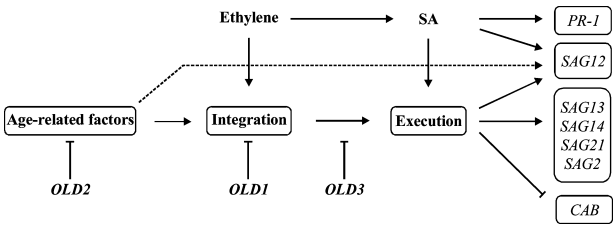

Figure 7. A genetic pathway showing the proposed positions of the *OLD* genes in the regulation of leaf senescence.

The model is constructed by analysing the phenotypes, genetics and *SAG* expression of the old mutants. The emphasis is on the control of *OLD* genes and their interaction with ethylene. An arrow indicates a stimulatory effect, while a T-bar represents an inhibitory effect. See text for details.

senescence varied largely, depending on the time when it was applied. Our results further confirmed the notion that ethylene can only induce senescence after a leaf reaches a certain developmental stage (Grbic and Bleecker, 1995; Weaver et al., 1998).

In extreme situations such as in the *ctr* plants in which ethylene signalling is constantly switched on, or in wild-type plants that are grown in the continuous presence of exogenous ethylene, early leaf senescence is not induced (data not shown, Kieber et al., 1993). In opposite situations such as in the *ein* mutants in which the ethylene signalling is permanently blocked, or in the plants with anti-sense constructs that reduce ethylene biosynthesis, normal leaf senescence eventually occurs (Bleecker et al., 1988; Grbic and Bleecker, 1995; John et al., 1995; Chao et al., 1997; Oh et al., 1997; Alonso et al., 1999). These observations exhibit that ethylene has no effect in stimulating senescence before or after certain developmental stages. In conclusion, a leaf has a defined age window to perceive the effect of ethylene on senescence, which is termed here as the senescence window.

Ethylene is involved in almost every façade of plant's development and responses to various stresses (reviewed by Johnson and Ecker, 1998). Ethylene response windows have also been described for other developmental events. Raz and Ecker (1999) showed that ethylene treatment does not affect the curvature of the apical hook in etiolated seedlings when given before or after certain growth stages. The effects of ethylene in releasing dormancy and promoting germination depend on the concentrations of exogenous ABA (Beaudoin et al., 2000). In tomato's susceptible response to pathogen infection, ethylene is required for the development of disease symptoms but is not essential for restricting pathogen infection and the primary lesion formation (Lund et al., 1998). In tomato, ethylene treatment can induce epinasty in the young and middle-aged leaves, but not in older ones (Abeles et al., 1992). Tomato fruit ripening was induced by exogenously-applied ethylene in mature green fruits, but not in immature fruits (Yang, 1987). These observations suggest that many such windows exist in plants to perceive ethylene action. When these windows open and how wide the windows are, may depend on the physiological context, developmental stage and the genetic make-up.

Many genes are involved in the biosynthesis, signalling and action of ethylene and are differentially regulated. In *Arabidopsis*, 5 genes encoding ACC synthase are differentially

expressed in plant organs, during development and in response to various stress conditions (Liang et al., 1992). Throughout plant development, the expression of the *ETR* ethylene perception gene family is differentially regulated in tomato (Lashbrook et al., 1998). In both *Arabidopsis* and tomato a large gene family of *EIN3* and *EIL* was identified (Chao et al., 1997; Tieman et al., 2001). Downstream are ethylene-response-factor1 and ethylene-responsive element binding factors whose differential regulation is also observed (Solano et al., 1998; Fujimoto et al., 2000). Such a sophisticated biosynthesis and signalling pathway is consistent with the existence of the diverse ethylene response windows.

The understanding of the relationship between the age-related factor and ethylene allowed us to formulate a model for the regulation of leaf senescence as shown in Figure 7. In this model, age-related factors are proposed to control the senescence window of a leaf and ethylene has to be recognised and integrated with the ageing signals. Perception of the signals leads to the activation of *SAG*s to accomplish senescence. This model provides clear implications: mutations causing perturbations in the control of age-related factors, in the integration of age and ethylene signals, or in the execution process of senescence may give predicted phenotypes. By utilising this knowledge and defining the senescence window, a screening system was established and mutants with altered leaf senescence phenotypes were isolated.

Functions of *OLD* genes

Bearing the model in mind, we tried to select those mutants in our collection with various senescence phenotypes that might locate at different positions in the regulating pathway. The phenotypes of the *old1*, *old2* and *old3* suggested that they potentially represented genes acting at the proposed positions (Figure 7). The physiological and molecular analyses were consistent with the suggested positions of the *OLD* genes, which were further confirmed by genetic interaction analysis.

The unique phenotype of *old2* suggests that in *old2* mutants the components of the regulatory pathway from ethylene signalling to the execution of senescence are not affected. Indeed, dark-grown *old2* seedlings showed a normal triple response. The *SAG* expression profile of ethylene-treated *old2* plants was not different from that of the wild-type, but advanced. Epistatic analysis also suggested that *OLD2* might work upstream of *OLD1* and *OLD3*. The simplest explanation could be that the *old2* mutation abolishes suppression on age-related factors and shifts the ethylene response window to an earlier stage. Thus, we infer that *OLD2* acts as a repressor of age-related factors upstream of ethylene action.

Air-grown *old1-1* plants display a strongly advanced senescence syndrome, which was further accelerated by ethylene treatment, suggesting that *old1* mutation generates alterations in two sets of pathways: age-regulated leaf senescence and ethylene signalling. This was more convincingly shown by the phenotypes of the *old1-1* and *old1-1etr1-1* double mutant. When ethylene perception was blocked in *old1-1*, the earlier onset of leaf senescence still occurred, but was not exaggerated by ethylene treatment. Thus, a plausible explanation for *old1-1* phenotypes would be that *OLD1* acts as repressor of the integration process.

It is important to notice that the *old1* mutants were different from *ctr* and *eto* that show a constitutive ethylene response (Guzmàn and Ecker, 1990; Kieber et al., 1993). *OLD1* is likely to define a novel link integrating ethylene actions into leaf senescence. Ethylene is shown to interact with many other signalling molecules such as with ABA in seed germination (Beaudoin et al., 2000), with jasmonic acid in conferring defence response (Penninckx et al., 1996), with

glucose in regulating seedling growth (Zhou et al., 1998), or with light in controlling hypocotyl elongation (Smalle et al., 1997). Isolation and analysis of *OLD1* may provide important insights on the interaction between senescence and ethylene signalling pathways.

In *old3*, leaf senescence occurred very rapidly, and ethylene treatment did not induce additional new leaves to senesce, suggesting that onset of leaf senescence in *old3* is independent of ethylene. We also constructed the *old3etr1-1* double mutants and observed that the lethal *old3* phenotype was not affected when the *etr1-1* mutation was introduced (data not shown). Therefore, *OLD3* may act downstream of ethylene at a late step of the senescence-regulating network with pleitrophic functions, which is consistent with the double mutant analysis that placed *OLD3* downstream of *OLD1*. One bottleneck of isolating early leaf senescence mutants is that mutations in homeostatic or housekeeping genes could also give an early senescence or lethal phenotype, which may not be distinguishable from the mutations in genes that specifically regulate leaf senescence. For instance, the premature senescence or lethal phenotype is generated from a mutation in a copper transport gene (Woeste and Kieber, 2000). It is hard to distinguish whether the lethal phenotype is the cause of the rapidly accelerated senescence or the consequence. Nevertheless, the lethality of *old3* can be prevented by culture in sucrose but the senescence is still accelerated in *old3*. This may argue against the first possibility. Together, we infer that *OLD3* may be a pleitrophic regulatory gene acting at a late step of the senescence-regulating networks.

In *Arabidopsis*, *SAG12* expression is highly associated with age-regulated senescence and is not induced by several stress conditions and believed to be a reliable marker for natural leaf senescence (Lohman et al., 1994; Weaver et al., 1998; Noh and Amasino, 1999). In *old1-1* and *old3*, *SAG12* consistently showed lower levels of mRNA upon ethylene treatment in comparison with the wild-type. These results raise two points. First, it is clear that high *SAG12* expression is not essential for the progression of leaf senescence in *old1-1* and *old3*. Second, these two *OLD* genes are likely to control a common pathway that is required but is not sufficient for the full expression of *SAG12* in wild-type plants. Disruption of *OLD2* alone did not induce *SAG12* expression. However, *old2* mutants showed higher levels of *SAG12* expression than *old1-1*, *old3* and the wild-type when treated with ethylene. This suggests that in *old2* mutants all the pathways required for full expression of *SAG12* are intact and active. In other words, *OLD2* controls an additional pathway that is involved in regulation of *SAG12* expression besides the *OLD1-OLD3* pathway. Therefore, analysis of *SAG12* expression in *old* mutants discloses at least two parallel regulatory pathways.

Previously, several factors required for the expression of *SAG12* have been identified. Noh and Amasino (1999) proposed the existence of a transcription factor that can bind to the senescence-specific promoter region of *SAG12* and initiate *SAG12* transcription in senescing leaves. Morris et al. (2000) showed that SA is also required for the expression of *SAG12*. The inducible *PR-1* expression demonstrated that the SA-mediated pathway is present in the *old* mutants. Thus *OLD* genes may be not involved in the SA-signalling pathway *per se*. Also, *OLD* gene products are unlikely to be the transcription factors only acting on *SAG12*, since a broad spectrum of SAG expression was altered. Nevertheless, it is clear that *OLD* genes are required for *SAG12* expression. Thus, we propose at least three different pathways to regulate *SAG12* expression in the model.

In summary, we have presented a model in an attempt to explain the control of senescence by age-related factors, the integration of ethylene action, and the regulation of SAG expression during the execution process. In contrast to the wealth of knowledge on the ethylene signalling

pathway, our current information on the molecular mechanisms of the regulation of leaf senescence and on the involvement of ethylene in senescence is scarce. Our model, although rudimentary, matches well the current knowledge available. The phenotypic, genetic and *SAG* expression analysis of the mutants provided information concerning at which steps the genes act and allowed for the construction of the model. Further refinements, through the isolation of mutants in additional steps, the analysis on double mutants from the *OLD* and the ethylene signalling pathways, and cloning and characterisation of the *OLD* genes, should yield more elaborate models for the regulation of leaf senescence.

Experimental procedures

Plant lines and growth conditions

Arabidopsis Landsberg *erecta* (L*er*-0) was the parental line for mutagenesis. Plants were grown in a growth chamber at 21°C and 70% relative humidity under ~60 $\mu molm^{-2}sec^{-1}$ fluorescent and incandescent light. Day length was set at 16h. An organic-rich soil (TULIP PROFI No.4, BOGRO B.V., Hardenberg, The Netherlands) was used for plant growth after sterilisation and drying. Plants for ethylene exposure were treated in a flow-through chamber at 20°C and ~50% relative humidity under continuous illumination. For experiments involving plants grown under sterile conditions, seeds were surface-sterilised with 25% commercial bleach and plated on Murashige and Skoog medium solidified with 0.8% agar.

Seed mutagenesis and isolation of mutants

Approximately, 20,000 seeds were treated with 0.3% ethyl methanesulfonate (EMS) for 15 h as previously described by Dijkwel et al. (1997). M_1 (mutagenised generation 1) seeds were sown on 500 pots of 20 cm in diameter, and seeds produced by 20-30 plants per pot were individually harvested and formed independent pools.

For each M_2 pool ~300 seeds were sown on a 20 by 30 cm tray. M_2 plants were grown for 11 d in air, treated with 10$\mu l\ l^{-1}$ ethylene (AGA, The Netherlands) for 3 d, and plants with yellowing cotyledons selected. The remaining plants were allowed to grow further in air for 7 d and treated with ethylene between 21-24 d, and plants with no signs of yellowing were sought. In total, ~150,000 plants were screened, and ~700 putative mutants were isolated. M_3 plants were further re-screened twice to eliminate false positives. Eventually, 62 mutants from 54 pools were obtained. The mutant lines were crossed twice to L*er*-0 before molecular and physiological characterisation.

Genetic analysis

For mapping, the *old* mutants were crossed to Col-0, and at least 30 F_2 plants homozygous for the *old* mutations were selected for DNA isolation using the SHORTY quick preparation (http://www.biotech.wisc.edu/*Arabidopsis*/). Linkage of the *old* mutations to cleaved amplified polymorphic sequences (CAPS) (Konieczny and Ausubel, 1993), simple sequence length polymorphism (SSLP) (Bell and Ecker, 1994) and single nucleotide polymorphsim (SNP) (Drenkard et al., 2000) markers was analysed.

To test the genetic interactions among the three *OLD* genes, *old1-1* pollen was used to pollinate *old2* and pollen from heterozygous *old3* plants was used to pollinate *old1-1* or *old2*. To check the segregation of the *old1-1* by *old3* cross, F_2 seedlings were grown in the aforementioned climate room for 14 d and scored for *old1-1* and the lethal *old3* phenotypes.

At 21 d the heterozygous *old3* seedlings were easily distinguished from the wild type. For segregation analysis of the *old1-1* by *old2* cross, F_2 seedlings were first grown in air, scored for *old1-1* phenotypes and subsequently transferred to ethylene chamber at 21 d. After a 3-d ethylene treatment, *old2* plants were with yellow cotyledons and 2-4 rosette leaves, which were distinguished from wild-type plants that showed yellow cotyledons plus 1 rosette leaf at the most. Similarly, the homozygous and heterozygous *old3* plants and the *old2* plants were scored in the F_2 progeny of the *old2* by *old3* cross.

To generate double mutants of *old1-1* and *etr1-1*, the *old1-1* pollen was used. The F_2 population was grown in soil and seedlings showing *old1-1* phenotypes were selected to check the presence of the *etr1-1* mutation using a PCR marker (Hua and Meyerowitz, 1998). Since *old1-1* was isolated from L*er*-0 and *etr1-1* was from Col-0, we also used the SNPs to genotype *old1-1etr1-1*. The F3 progeny was used for experiments.

Measurements of chlorophyll content and ion leakage

Individual leaves or whole rosettes were taken from at least 6 seedlings. For measuring the chlorophyll content, samples were incubated overnight in 80% (v/v) aceton at 4°C in darkness, and the chlorophyll content quantified spectrophotometrically using the method of Inskeep and Bloom (1985). For measuring ion leakage, leaf samples were immersed into deionised carbonate-free water, shaken in a 25°C water bath for 30 min, and the conductivity was measured using a Wissenschaftlich Technische Werkstatten conductivity meter (model KLE1/T, , Weilheim, Germany). Then samples were boiled for 10 min and conductivity measured again. The percentage of the first measurement over the second measurement was used as the membrane damage indicator.

RNA extraction and Northern blot analysis

Plant material was frozen in liquid nitrogen and total RNA was isolated and quantified spectrophotometrically. For RNA blot analysis, 5 or 10 µg of RNA samples were separated by electrophoresis on a 1.2% agarose gel containing 1.8% formaldehyde and then capillary-blotted onto Hybond-N^+ nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, England). Ethidium bromide was included in the sample-loading buffer at a concentration of 4 µg/ml to confirm equal sample loading and blotting. Probes described in Table 2 were ^{32}P labelled by random-priming, and hybridisation was done at 65°C overnight in the Church and Gilbert (1984) buffer, and the membranes were washed once in 2x SSC for 5 min and twice for 15 min each in 0.1x SSC and 0.1% SDS at 65°C. Probe hybridisation was visualised with a phosphorimager using the OptiQuant software (Canberra Packard, Zellik, Belgium). Stripping of the membranes was done by boiling in 0.5% SDS solution for 3 min, and each membrane was used for probing maximally three times.

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Chapter 3
***Arabidopsis onset of leaf death* mutants reveal a complex regulation of ethylene-induced senescence**

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Abstract
The effect of ethylene exposure time on leaf senescence was studied in *Arabidopsis* accessions L*er*-0, Col-0 and Ws-0 and in several *old* (*onset of leaf death*) mutants representing different genetic loci. Leaf senescence was observed in 24-day-old plants exposed to ethylene for 3 to 16 days. For ethylene treatments up to 12 days, a longer ethylene exposure time resulted in more senescing leaves in wild type plants. However, a 16-day ethylene treatment reduced senescence symptoms. *old* mutants differentially responded to varied ethylene exposure times. In *old3, old9, old12* and *old14* mutants, the same number of leaves turned yellow, regardless of the ethylene exposure time. In *old5* and *old13*, increasing ethylene exposure time from 6 to 16 days resulted in the same number of yellow leaves, but less leaves yellowed in a 3-day treatment. The amounts of senescing leaves in *old1-1* and *old11* mutants decreased with increases in ethylene exposure time from 6 to 16 days. Thus, multiple *OLD* genes may act to restrict the induction of senescence by ethylene in an ethylene exposure time-dependent manner. The data substantiate the notion that there is a complex interaction between leaf age and ethylene involving multiple genetic loci.

Keywords: Leaf senescence, *Arabidopsis* accessions, ethylene, *old* mutants

Introduction
The phenomenon of senescence can be appreciated among deciduous trees and in the ripening of cereal crops during late summer and autumn. This occurs at a global scale and changes the appearance of the Earth from space. An estimation based on the records of orbiting satellites states that over the entire globe, about 1.2 billion tonnes of chlorophyll and 200 million tonnes of carotenoids are degraded annually (Hendry, 1988). The colour changes during senescence are associated with active physiological and biochemical processes, such as dismantling of chloroplasts, degradation of RNA and proteins, and translocation of macro-/micro-molecules from senescing leaves to other parts of the plant (Bleecker and Paterson, 1997). Senescence requires active transcription of many senescence-associated genes (*SAG*s), which have been identified in a variety of species including *Arabidopsis*, rapeseed, rice and maize. Detailed studies on the *SAG* identities and the expression patterns suggest that a complex network regulates leaf senescence (For reviews, see Smart, 1994; Buchanan-Wollaston, 1997; Gan and Amasino, 1997; Nam, 1997; Gan, 2003).

Currently, at least three approaches are being taken to study the regulation of leaf senescence. First, it is believed that leaf senescence may be not only associated with, but also driven by *SAG*s and that leaf senescence can be altered by manipulation of the *SAG* activities (Gan and Amasino, 1997; Gan, 2003). One successful example of this approach is provided by the identification of *SAG101* that delayed senescence when its expression was antisense-suppressed, but promoted senescence when it was overexpressed (He and Gan, 2002).

The second approach is based on the hypothesis that factors regulating the expression of *SAGs* are important for the control of leaf senescence. Common regulatory components may be identified through comparing the expression profiles of *SAGs* during development and under various senescence-inducing conditions (Weaver et al., 1998; He et al., 2001; Buchanan-Wollaston et al., 2003; Gepstein et al., 2003). Senescence is often initiated once a leaf reaches full expansion and maturity, indicating that the primary regulators of senescence are endogenous, age-related signals (Hensel et al., 1993). Internal physiological and biochemical

processes, important for the initiation of leaf senescence, include changes in the photosynthetic productivity (Hensel et al., 1993; Thomas and Howarth, 2000) and plant endogenous hormones (Gan and Amasino, 1995; Grbic and Bleecker, 1995; Morris, et al., 2000; He et al., 2002; Yin et al., 2002). Furthermore, external stress conditions such as drought, light deprivation, oxidative stress, acute and chronic ozone fumigation, and pathogen challenge, can accelerate leaf senescence as well (Park et al., 1998; Weaver et al., 1998; Pointer et al., 1999; Navabpour et al., 2003). By comparing the expression profiles of *SAGs* upon various induction conditions, these studies have helped clarify which factors are more effective in inducing expression changes of particular subsets of *SAGs*, and allowed the construction of a regulating network of *SAG* expression (Buchanan-Wollaston et al., 2003).

Mutational analysis is the third approach employed to identify genetic loci that are involved in the regulation of leaf senescence. Mutants that can stay green longer have been paid much attention due to the potential relevance to the improvement of crop yields. Five different ways of 'stay-green' have been described to facilitate the search for genetic loci or physiological processes that underlie the phenotype (Thomas and Howard, 2000). The stay-green mutants have been isolated in a variety of crop plants including wheat (Spano et al., 2003), rice (Cha et al., 2002), *Lolium* (Thomas et al., 2002), and soybean (Guiamet et al., 2002; Luquez and Guiamet, 2002). By visually observing the yellowing of detached leaves incubated in the dark, several classes of *Arabidopsis ore* mutants that exhibited delayed leaf senescence were isolated (Oh et al., 1997). Similarly, dark-induced whole-plant senescence was used to isolate accelerated or delayed leaf senescence mutants (Yoshida et al., 2002a; 2002b). *ORE9* and *DLS* were shown to encode components of the ubiquitin-mediated protein degradation pathway (Woo et al., 2001; Yoshida et al., 2002b), *ORE4* encodes a plastid ribosomal protein (Woo et al., 2002), and *CPR5/HYS1* encodes a plant specific protein with pleiotropic functions (Bowling et al., 1997; Kirik et al., 2000; Yoshida et al., 2002a; Chapter 4). Thus, observing dark-induced visible yellowing is an efficient approach to pinpoint senescence regulatory genes in *Arabidopsis*.

Since discovered as the active component in the leaks of gas from street lights in 1864 that caused stunting of growth, twisting of plants, and abnormal thickening of stems (the triple response) (Neljubow, 1901), ethylene has been shown to be a native gaseous product of plants and plays essential roles in many aspects of plant growth and development (Johnson and Ecker, 1998). The involvement of ethylene in leaf senescence has been addressed in several studies, in which a fixed length of ethylene treatment (e.g. 3 days) was applied to plants throughout development (Grbic and Bleecker, 1995; Weaver et al., 1998; Jing et al., 2002). This experimental approach addressed how leaf age determined the effect of ethylene on senescence and has provided solid evidence for the notion that ethylene only works to promote senescence after leaves are primed to senesce (Grbic and Bleecker, 1995; Weaver et al., 1998). Based on these studies, a senescence window concept was proposed to describe the relationship between leaf age and ethylene, which split leaf development into three distinct phases depending on whether and how senescence can be induced (Jing et al., 2003). This concept emphasises the developmental control of leaf senescence and considers leaf age as an ultimate determinant of senescence progression.

However, senescence could not be induced if plants were treated with ethylene for a short period, even if a leaf was primed for senescence (Weaver et al., 1998; Jing et al., 2002). These observations imply that a certain minimal ethylene exposure time is required to induce leaf senescence. Prompted by these observations, we attempted to further address the relationship between leaf age and ethylene and examined how the effect of ethylene on senescence might

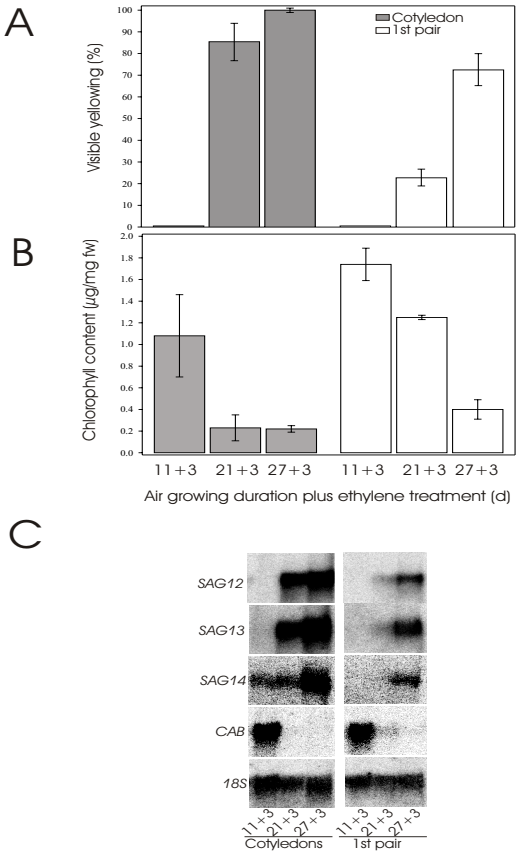

Figure 1. Comparison of ethylene-induced senescence in cotyledons and rosette leaves. L*er*-0 plants were grown first in air for 11, 21, or 27 days, and then exposed to ethylene for 3 days. The visible yellowing (**A**) of cotyledons and the first pair of rosette leaves was subsequently scored as the percentage of yellow cotyledons or rosette leaves *versus* the total number of cotyledons or rosette leaves, respectively. The chlorophyll contents (**B**) and mRNA levels of several senescence-associated genes (**C**) were subsequently analysed. For each time point, the observations on visible yellowing were on 3 sets of 50 plants and the results for chlorophyll were shown as mean±sd of four replicates. Total RNA was isolated from leaf samples of approximately 100 plants. Five-µg total RNA was used for Northern blotting. The membrane was sequentially hybridised with cDNA probes of the indicated *SAG*s, *CAB* and rRNA.

be influenced by the time of ethylene exposure. For this, leaf senescence was examined in plants with the same final age but varied ethylene exposure time. Ethylene-induced senescence was compared between wild-type plants and *old* mutants and the results revealed a complex interaction between leaf age and ethylene involving multiple genetic loci.

Results

Correlation between ethylene-induced visible yellowing and senescence-associated physiological and molecular processes

We examined the effects of ethylene in inducing leaf senescence by monitoring changes of senescence-associated morphological, physiological and molecular markers in cotyledons and rosette leaves. As shown in Figure 1A, visible yellowing was observed in 21+3 (21 days in air plus 3 days in ethylene) and 27+3 plants but not in 11+3 plants. The visible yellowing induced by ethylene was more pronounced in cotyledons than in the first pair of rosette leaves, and in 27+3 samples than in 21+3 samples. The chlorophyll content was the highest in the 11+3 samples, corresponding to no visible yellowing in these samples, and dropped in the 21+3 and 27+3 samples (Figure 1B). The decrease in chlorophyll content was more pronounced in cotyledons than in the first pairs of rosette leaves. These patterns were associated with

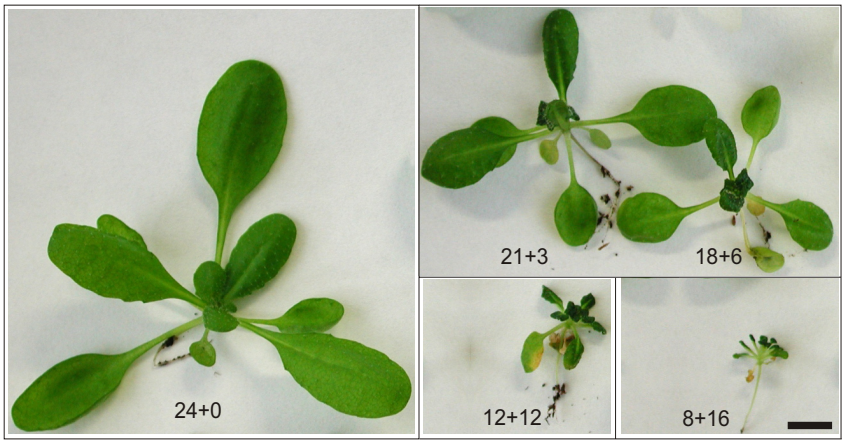

Figure 2. Representative L*er*-0 plants grown in air or exposed to ethylene for various periods. Plants were grown either in air for 24 days, or first in air for 21, 18, 12, or 8 days and then exposed to ethylene for 3, 6, 12, or 16 days, respectively. At the end of the treatments (24 days), representative plants were selected and photographed. The bar represents 0.5cm.

Table 1. Total leaf numbers of *Arabidopsis* accessions and *old* mutant lines after various ethylene treatments*

Lines	21+3	18+6	12+12	8+16
L*er*-0	12.7 ± 0.24	13.3 ± 0.24	15.1 ± 0.24	15.1 ± 0.35
Col-0	13.3 ± 0.14	14.1 ± 0.42	15.5 ± 0.06	16.0 ± 0.41
Ws-0	12.1 ± 0.14	12.6 ± 0.30	13.8 ± 0.23	13.6 ± 0.06
ctr1-1	11.8 ± 0.07	11.8 ± 0.21	13.0 ± 0.49	13.0 ± 0.14
old1	11.0 ± 0.47	11.0 ± 0.37	10.9 ± 0.12	11.0 ± 0.12
old5	12.6 ± 0.22	13.3 ± 0.08	14.4 ± 0.30	13.9 ± 0.16
old14	11.5 ± 0.22	11.30 ± 0.04	11.1 ± 0.28	11.4 ± 0.21
old9	12.7 ± 0.25	12.7 ± 0.14	13.7 ± 0.06	14.5 ± 0.06
old11	12.6 ± 0.27	12.9 ± 0.14	13.4 ± 0.20	12.9 ± 0.14
old13	12.1 ± 0.28	12.4 ± 0.27	14.0 ± 0.24	13.8 ± 0.29
old3	4.0 ± 0	4.0 ± 0	4.0 ± 0	4.0 ± 0
old12	11.5 ± 0.04	11.5 ± 0.12	12.1 ± 0.03	12.3 ± 0.41

*Plants were grown and treated with ethylene as described in Experimental procedures. A leaf was scored when it emerged and was visible (over 1mm). Scoring was performed on 24-d-old plants at the end of the ethylene treatment. Approximately 30 plants were used for each line.

decreases in the mRNA levels of *CAB* (Chlorophyll a/b binding protein) (Figure 1C). The mRNA levels of *SAG12*, *SAG13* and *SAG14*, which are believed to be good molecular markers for the progression of leaf senescence (Lohman et al., 1994; Weaver et al., 1998), were induced by a 3-d ethylene treatment in 21+3 samples, and to higher levels in 27+3 samples (Figure 1C). Nevertheless, no induction of *SAG12* and *SAG13* expression by ethylene was observed in the youngest (11+3) samples. Thus, the effects of ethylene in inducing visible yellowing correlated with changes in chlorophyll content and in the *SAG* mRNA levels. We continued to examine the control of ethylene-induced leaf senescence using visible yellowing as a parameter.

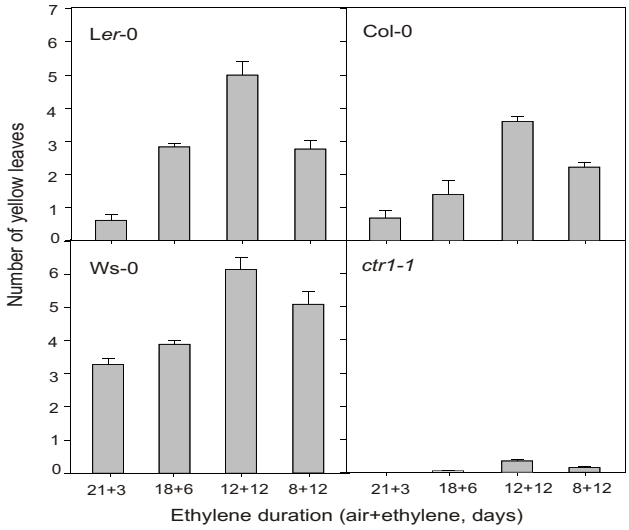

Figure 3. Visible yellowing of 24-d-old plants of three *Arabidopsis* accessions and *ctr1-1* mutants exposed to ethylene for various amounts of time.
Plants of L*er*-0, Ws-0, Col-0 and *ctr1-1* were grown first in air for 21, 18, 12, or 8 days, and then exposed to ethylene for 3, 6, 12, or 16 days, respectively. The visible yellowing was subsequently scored and expressed as means±sd of at least three replicates of 30 plants each.

Similar and different responses of *Arabidopsis* accessions to ethylene

The effect of ethylene in inducing visible yellowing was studied by employing a constant final age/ variable induction experimental design, such as described by Weaver et al. (1998). The *Arabidopsis* accessions L*er*-0, Col-0 and Ws-0 were grown in air for 8, 12, 18 and 21 days, and then treated with ethylene for 16, 12, 6 and 3 days, respectively. This resulted in plants with the same final age of 24 days that were exposed to ethylene for varied duration. The constitutive ethylene response mutant *ctr1-1* (Kieber et al., 1993) was included as a control that mimics a continuous ethylene treatment. Ethylene can inhibit cell elongation, reduce leaf and plant size and promote flowering (Johnson and Ecker, 1998). We hence examined whether the effects of changing ethylene exposure time on senescence correlated with its effects on plant growth. Figure 2 shows representative L*er*-0 plants following the various ethylene treatments. Clearly, the longer the ethylene treatments, the smaller the leaf and plant sizes. The plants in the 12+12 and 8+16 treatments became stunted with compacted rosettes and epinastic leaves, phenocopying the *ctr1-1* mutant. Similar phenotypes were observed in Col-0 and Ws-0 (data not shown). The average total leaf numbers in plants with various ethylene treatments were compared and the results showed that plants of the three accessions that experienced a longer ethylene exposure had more leaves (Table 1). Thus, the effects of ethylene in the inhibition of cell elongation and promotion of development increased with the increase in ethylene exposure time.

Figure 3 presents the results of visible yellowing scored for the 24-d-old plants. A common trend was observed in the three accessions. With an increase in ethylene exposure time from 3 to 12 days, the number of leaves exhibiting visible yellowing increased. However, when the ethylene exposure time was further increased to 16 days, a reduction in visible yellowing was observed. Remarkably, such a pattern of visible yellowing was similarly observed in *ctr1-1*, although the number of leaves that were visibly yellow was much less (Figure 3). This is different from the effect of ethylene on growth and development where the longest treatment

Table 2. Numbers and distribution of *old* mutants with early leaf senescence

Mutant phenotypes	L*er*-0	Col-0
Class I Enhanced yellowing in air and after ethylene exposures	30	13
Class II Enhanced yellowing only after ethylene exposure	11	-
Class III Enhanced yellowing in air, but no additional yellowing after ethylene exposure	7	-

did not result in a decrease in total leaf number. If the same period of ethylene treatment (e.g. 3 or 6 days) was applied to plants with a final age of 12, 18, or 21 days, more yellow leaves were observed in older plants (Jing et al., 2002; data not shown). Thus, when a fixed term of ethylene treatment is applied to different developmental stages, leaf senescence is primarily determined by leaf age, whereas in the case of a fixed final leaf age, the length of the ethylene treatment time can affect the induction of senescence.

Besides the common trend, variation in the responses to ethylene was observed in the three accessions. In all the treatments, Ws-0 plants exhibited the highest amounts of yellow leaves. Indeed, yellow cotyledons were observed in Ws-0 plants with a final age of 11 days that were treated for 3 days with ethylene, but not in similarly treated L*er*-0 and Col-0 samples (data not shown). Thus, Ws-0 appeared to be more responsive than L*er*-0 and Col-0 to ethylene's induction of visible yellowing. Furthermore, L*er*-0 exhibited higher amounts of yellow leaves than Col-0 in most cases.

In summary, we conclude that ethylene influences the visible yellowing of a leaf in a different way as it affects other aspects of plant development. The induction of leaf senescence by ethylene is strongly influenced by ethylene exposure time.

Arabidopsis old mutants with altered leaf senescence

Sixty-one *onset of leaf death* (*old*) mutants were isolated from EMS mutagenised populations of L*er*-0 and Col-0 seeds and were classified into 3 subgroups (Table 2; Jing et al., 2002). Two remarkable differences were observed between the L*er*-0 and Col-0 mutant populations. Using the same mutant selection criteria, more mutants were isolated from L*er*-0 than from Col-0. Furthermore, L*er*-0 early leaf senescence mutants were distributed among the three classes, whereas the Col-0 ones all belonged to Class I. The observed differences may reflect the variation in the genetic control of senescence between the two accessions.

Several L*er*-0 mutants were selected for phenotypic and genetic characterisation (Figure 4; Table 3). The selected Class I mutants showed early senescence symptoms in air (Compare Figures 4A to C, E, G, I, K), and the symptoms were further enhanced by ethylene treatment (Compare Figures 4B to D, F, H, J, L). Early senescence was observed after ~11-12 days in *old4*, *old7* and *old10*, but after 16 days in *old5* and *old14*. Yellowing of air-grown *old4* was associated with lesion formation. The *old4, old5, old10* and *old14* mutants did not markedly differ from wild type plants, other than the altered senescence phenotype. Adult *old7* plants, however, were smaller than the wild type. Air-grown Class II mutants were not different from the wild type when grown under our standard conditions, but displayed enhanced senescence symptoms following ethylene treatment (Figures 4M, N, O). In *old13*, ethylene-induced senescence was associated with lesion formation. Two *old12* alleles belonging to the third Class mutants whose advanced senescence symptoms are not further enhanced by ethylene exposure, were isolated (Figure 4P).

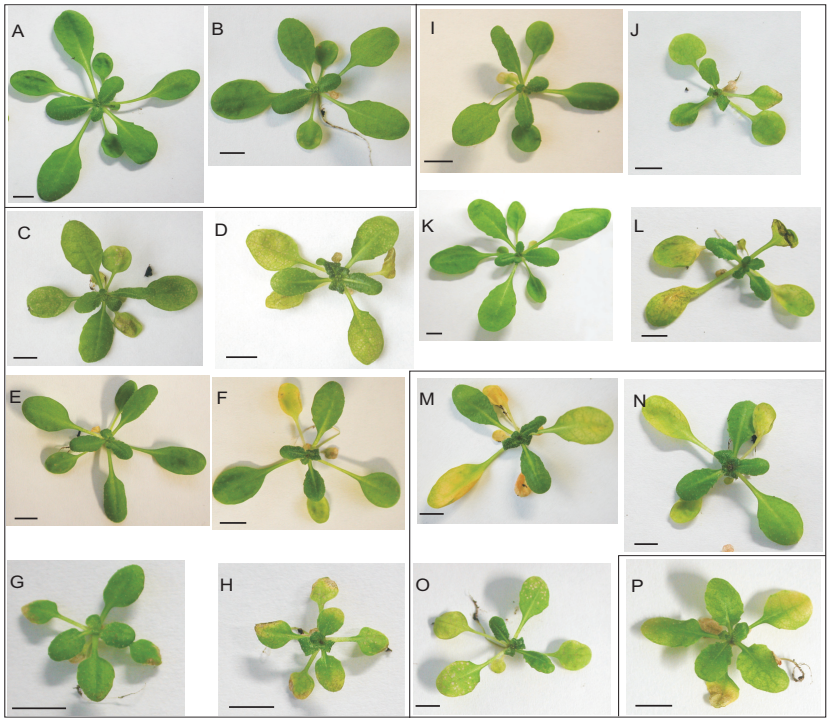

Figure 4. Phenotypes of 24-d wild type plants and *old* mutants with or without a 3-d ethylene treatment. (**A-L**) Representative air-grown or ethylene-treated L*er*-0 (**A-B**), *old4* (**C-D**), *old5* (**E-F**), *old7* (**G-H**), *old10* (**I-J**), and *old14* (**K-L**) plants.
(M-**O**) Ethylene-treated *old9*, *old11*, and *old13* plants.
(**P**) Air-grown *old12* plants.
The bars in the graphs represent 0.5cm.

Genetic analyses showed that the characterised *old* alleles segregated as monogenic recessive traits except *old9* and *old11*, which segregated as conditional (ethylene-dependent) co-dominant traits (Table 3). Allelism tests between mutants within each class revealed that they belong to different complementation groups (data not shown). Genetic mapping placed *old12* and *old9* on chromosome 2 at ~60cM (centiMorgan) and ~80cM, respectively; *old14* on chromosome 3 at ~70 cM; and *old11*, *old5* and *old13* on chromosome 5 at ~60cM, 75cM and 110cM, respectively. Thus, these *old* mutants represent multiple genetic loci.

Differential responses of *Arabidopsis old* mutants to ethylene

The effect of variable lengths of ethylene treatment on the induction of visible yellowing of 24-d *old* mutants was determined. The previously described *old1-1* and *old3* mutants were included and the results are presented in Figure 5. All the mutants responded to the ethylene treatments in a different way than the wild type. In the *old3*, *old9, old12,* and *old14* mutants, a similar number of yellow leaves was observed in all the treatments, indicating that these mutants become insensitive to the changes in ethylene exposure time once exposed to ethylene for at least 3 days. The *old5* and *old13* mutants showed a similar pattern except that the

Table 3. Genetic segregation of *old* genes

Class	Male	Female	Generation	Wild-type	Mutant	χ^{2c}
I[a]	*old4*	L*er*-0	F_1	10	0	
			F_2	238	87	0.54
	old5	L*er*-0	F_1	31	0	
			F_2	151	64	2.61
	old7	L*er*-0	F_1	7	0	
			F_2	411	116	2.51
	old10	L*er*-0	F_1	6	0	
			F_2	118	53	3.28
	old14	L*er-0*	F_1	39	0	
			F_2	156	40	2.20
II[b]	*old9*	L*er*-0	F_1	0	49	
			F_2	18	73	1.32
	old11	L*er*-0	F_1	0	34	
			F_2	19	67	0.75
	old13	L*er*-0	F_1	52	0	
			F_2	180	79	4.18
III[a]	*old12*	L*er*-0	F_1	24	0	
			F_2	328	87	3.61

[a]The Class I and Class III mutants showed early senescence when grown in standard growth conditions. Thus, the scoring of phenotypes was performed before ethylene treatment and plants with clearly visible yellowing cotyledons and/or rosette leaves were scored as mutants.

[b]The segregation analysis for Class II mutants was performed after ethylene treatment. The phenotype scoring was carried out on 21-d-old plants treated with ethylene for 3 days. The criteria were: wild-type plants with no yellow leaves, Class II mutants with at least 3 yellow leaves including cotyledons.

[c]All the χ^2 values were calculated for the 1:3 segregation ratios of mutants:wild type except in the case of *old9* and *old11*, where a 3:1 ratio of mutants:wild type was calculated.

shortest ethylene treatment (21+3) caused a lower level of visible yellowing, implying that these two mutants may require a longer minimal ethylene exposure time. Similar to the wild type, the *old1-1* and *old11* mutants did not show the maximum number of yellow leaves with the longest ethylene treatment. Here, the maximal level of visible level was achieved with the 21+3 or 18+6 treatment, instead of the 12+12 treatment in the wild type plants. Interestingly, the maximal number of yellow leaves induced by the various ethylene treatments was between 5 and 7 in both the wild type L*er*-0 and the *old* mutants, the only exception being the *old3* mutant which is seedling-lethal (Jing et al., 2002).

The effect of 3 to 16 day ethylene treatment on the development of the *old* mutants was measured (Table 1). Remarkably, the *old1-1, old11,* and *old14* mutants did not show the increases in the total rosette leaf numbers with the increased ethylene duration as observed in the wild type plants (Table 1), suggesting that other ethylene-dependent processes are affected as well. Overall, the *old* mutants seem to have different requirements for ethylene exposure time, implicating that the *OLD* genes may act to regulate the senescence response of plants to the ethylene exposure time.

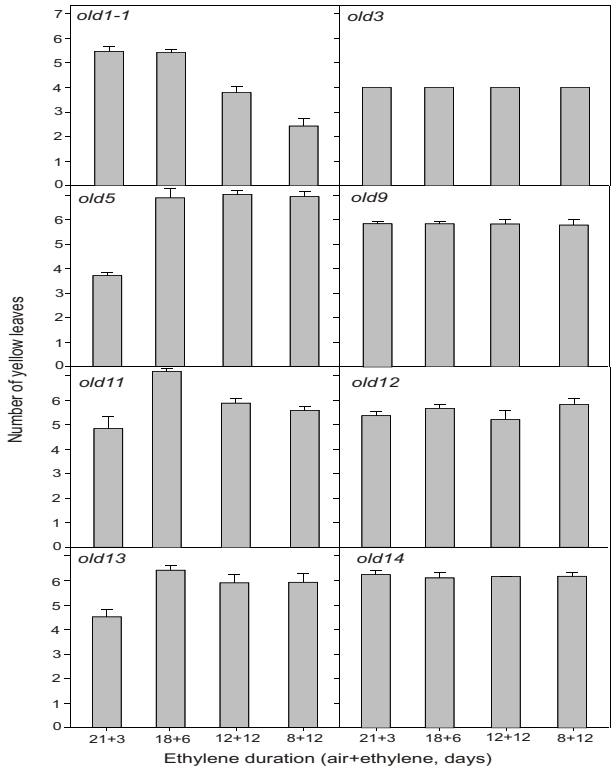

Figure 5. Visible yellowing of 24-d-old *old* mutants exposed to ethylene for various amounts of time.
The *old* mutants were grown first in air for 21, 18, 12, or 8 days, and then exposed to ethylene for 3, 6, 12, or 16 days. The visible yellowing was subsequently scored and expressed as means±sd of at least three replicates of 30 plants each.

Discussion

The onset of leaf senescence depends on the interaction between leaf age and ethylene exposure time

Molecular genetic and biochemical studies have revealed that ethylene biosynthesis, perception, signalling and effectors are controlled by gene families, which are differentially expressed among organs, throughout development, and upon induction (Abeles et al., 1992; Johnson and Ecker, 1998; Klee and Tieman, 2002; Chang and Bleecker, 2004). This multitude and complexity of genes is consistent with the many functions of ethylene in plants. Theoretically, every plant cell has all these components and is able to allow all the ethylene actions regardless of the origin of the cell. Clearly, other genetic components are required to differentially regulate ethylene action. As a developmental event, leaf senescence consistently appears on leaves that are matured to a certain age and progress in a leaf-age dependent manner. This particular feature promoted people to assume that in plants there are specific age-related factors or death signals, which modulate various internal and external cues to control leaf senescence (reviewed by Nooden et al., 1997). Ethylene has been shown to only promote senescence in leaves that are matured to a certain age (Grbic and Bleecker, 1995; Weaver et al., 1998; Jing et al., 2002). Hence, it is possible that there are specific interactions between age-related factors and ethylene to determine how and when ethylene promotes senescence. Here, we explored the effect of ethylene exposure time on the induction of

senescence and stimulation of plant development. Under our experimental conditions, increased ethylene treatments with a length of up to 12 days, showed increased effects in the induction of senescence. However, a further increase in ethylene exposure time reduced the senescence symptoms. These findings were demonstrated in three *Arabidopsis* accessions, although the amount of leaves in which senescence was induced differed between the accessions. Thus, it seems that long ethylene exposure times can somehow desensitise leaves for the effect of ethylene on senescence. Intriguingly, a similar pattern was observed in *ctr1-1*, implicating that this mutant is still able to respond to exogenously applied ethylene in a manner similar to the wild type. Nevertheless, the results are consistent with the phenotype of *ctr1-1* mutants: even though the ethylene signalling pathway is continuously switched on, senescence does not occur much faster than in the wild type (Kieber et al., 1993). The effect of ethylene exposure time on plant development was different. Here, an increase in ethylene exposure time caused an increase in the speed of development. Similarly, cell expansion was inhibited in young emerging leaves, while senescence could not be induced in the youngest leaves. This implicates that induction of senescence by ethylene can occur independently from other ethylene-dependent events.

Thus, the results presented here are in agreement with the existence of the proposed age-related factors. Such factors are strictly dependent on age, but can be modulated by ethylene exposure time.

Multiple genetic loci control ethylene-induced senescence

The effect of leaf age and ethylene on senescence is under genetic control. This was supported by the observed variation in ethylene-induced senescence of different *Arabidopsis* accessions. In fact, the Ws-0 accession could be considered as an "early senescence mutant" as compared to the L*er*-0 and Col-0 accessions. Furthermore, Col-0 was less responsive than L*er*-0. This natural variation seems to be associated with the involvement of multiple genetic loci, as a higher number of *old* mutants were isolated from L*er*-0 than from Col-0. We have also observed that in Col-0, Ws-0 and 9 additional accessions, *old3*-induced senescence segregated different than in L*er*-0 and 4 other accessions (Jing et al., 2002; Chapter 5). Such accession-dependent differences could be related to the differential regulation of ethylene synthesis as well. *Arabidopsis* Ws-0 accession was shown to be more sensitive to ozone injury than Col-0, which was associated with increased ethylene synthesis in Ws-0 (Tamaoki et al., 2003). Natural variations have been observed for a variety of life history traits in *Arabidopsis* including the control of flowering time (Koornneef et al., 1998), disease resistance and tolerance (Kover and Schaal, 2002), and the control of cytosine methylation in the nucleolus organiser regions (Riddle and Richards, 2002).

The isolation of *old* mutants representing multiple genetic loci further confirmed the genetic basis for the regulation of ethylene-induced senescence. In addition to the previously described *old1*, *old2* and *old3* mutants (Jing et al., 2002), here we present 9 additional mutants, which exhibited various enhanced leaf senescence phenotypes as compared to the wild-type. Phenotypic and genetic analyses confirmed that these mutants represent different genetic loci.

Remarkably, the maximum number of leaves that could become yellow did not differ greatly between the wild type and the mutants. The L*er*-0 accession showed 5 yellow leaves after 12 days of ethylene treatments. This is similar to the *old1-1* mutant after 3 or 6 days of ethylene exposure. The *old9*/*old12*/*old13*/*old14* and *old5*/*old11* mutants showed a maximum of 6 and

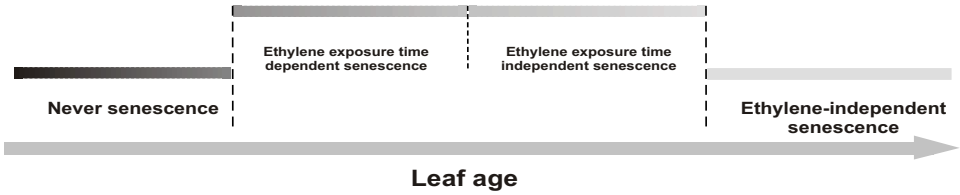

Figure 6. A model depicting the possible four different phases of the senescence window. See discussion for details.

7 yellow leaves, respectively. Thus, emerging leaves strongly suppress the effect of ethylene on senescence in the wild type as well as in the mutants. The mutants were divided into three different classes, based on their phenotypes after growth in air and after a 3-d ethylene treatment. The mutants could be further divided into different groups, depending on their phenotype after long ethylene treatments. For instance, *old1-1*, *old5* and *old14* were classified as Class I mutants, but exhibited different responses to increases in ethylene exposure time. Furthermore, *old3*, *old9, old12* and *old14* belong to three different classes but showed a similar pattern in response to the changes in the ethylene time. The extent and diversity of the *old* mutants suggest a complex regulatory network that controls age- and ethylene-induced senescence.

It was proposed that during leaf development each individual leaf goes through 3 different phases (Jing et al., 2003). In the first 'never-senescence phase' ethylene is unable to induce senescence, while the second 'ethylene-dependent phase' allows the induction of senescence by ethylene. The third phase marks the age-dependent, but ethylene-independent induction of senescence. Based on this proposed senescence window concept, genes important for senescence regulation are those that control the phase transitions of individual leaves. Our previous study indicated the *OLD1*, *OLD2* and *OLD3* genes may regulate the first transition from the never-senescence phase to ethylene-dependent senescence phase (Jing et al., 2002). Here, we observed that in wild type plants, increasing ethylene exposure time gradually increased the amounts of yellow leaves suggesting that this very first phase transition is ethylene exposure time dependent, or that there is an ethylene exposure time dependent phase (Figure 6). In all the isolated *old* mutants, the responsiveness to the increase in ethylene exposure time was altered, suggesting that these *OLD* genes may act to restrict the induction of senescence by ethylene in an ethylene duration-dependent way. The results, therefore, are consistent with the existence of such a proposed phase. The separation of the different phases of senescence development may help to define the acting points of the senescence regulating genes. For instance, the senescence phenotypes of *old3, old9, old12* and *old14* were not affected by changing ethylene exposure time, suggesting that these genes may control the ethylene duration-dependent phase. Our previous study suggested that *OLD1* works to control the integration of age and ethylene signal (Jing et al., 2002). This was further supported by the results obtained in this ethylene duration study since the longer the exposure time, the less the senescence symptoms in *old1-1* plants. This suggests that *old1-1* plants are hyper-responsive to ethylene in an age-dependent manner.

Taken together, the findings in this study show the involvement of multiple gentic loci in the control of ethylene-induced senescence. Further cloning and characterisation of *OLD*

genes, together with the exploration of natural variation in *Arabidopsis*, will help identify the molecular mechanisms that regulate leaf senescence.

Experimental procedures

Plant material and growth conditions

Arabidopsis Landsberg *erecta* (L*er*-0), Columbia (Col-0) and Wassilewskija (Ws-0) were used in this study. The growth conditions used were essentially as described by Jing et al. (2002). For the experiments described in Figure 1 an organic-rich soil (TULIP PROFI No.4, BOGRO B.V., Hardenberg, The Netherlands) was used. For the other experiments γ-ray radiated soil was used (Hortimea Groep, Elst, The Netherlands). The ethylene dosage was approximately 10µl/l since it has been shown that a dosage ranging from 1-100µl/l was sufficient to generate the same effect on senescence (Chen and Bleecker, 1995; Sakai et al., 1998).

Seed mutagenesis, mutant screening and genetic analyses

Generation of M_1 and M_2 seeds, mutant screening, genetic segregation, and linkage analyses between *old* mutants and PCR-based molecular markers were described previously (Jing et al., 2002).

Visible yellowing observation, chlorophyll content measurement, and Northern blot analyses of RNA

Cotyledons or rosette leaves that were yellow over an area of more than 5% of the leaf blade were judged as yellow as suggested by Lohman et al. (1994). However, in our experiments, yellowing could be initiated at the leaf tips, at the petiole side of the leaf, or in the middle of the leaf blade, which did not always resemble the developmental yellowing pattern of leaf senescence. The experimental procedures for chlorophyll content measurement and Northern blot analysis of RNA were described by Jing et al. (2002).

Acknowledgements

We would like to thank Bert Venema and Otto Lip for their excellent technical supports. We also thank the *Arabidopsis* Biological Resource Centre for providing *Arabidopsis* seeds.

Chapter 4
***Arabidopsis CPR5* is a senescence regulatory gene that exhibits early-life beneficial but late-life deleterious effects**

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Abstract
Arabidopsis cpr5 mutants exhibit multiple phenotypes including enhanced pathogen resistance, abnormal trichome development, spontaneous lesion formation and accelerated leaf senescence, indicating that *CPR5* is involved in multiple facets of the *Arabidopsis* life cycle. This study aimed to analyse the functions of *CPR5* during development. Double mutants between *cpr5* and hormonal mutants as well as transgenic plants with different *CPR5* mRNA levels were constructed and studied. The *cpr5* mutants exhibited enhanced responses to multiple signalling molecules and revealed complex interactions among salicylic acid, jasmonic acid and ethylene. The spontaneous lesion formation and early leaf senescence phenotypes of *cpr5* mutants required discrete hormonal signals and could be uncoupled in different genetic backgrounds. Ectopic expression of *CPR5* restored all the mutant phenotypes in plants up to the bolting stage. At late development, however, accelerated leaf senescence could be envisaged by the earlier occurrence of visible yellowing and increased levels of *SAG12* mRNA. Thus, *CPR5* has early-life beneficial effects by repressing cell death but late-life deleterious effects by promoting developmental senescence. As such, CPR5 appears to function as a typical senescence regulatory gene predicted by the evolutionary theories of senescence.

Keywords: *Arabidopsis,* leaf senescence, evolutionary senescence, hormones, *CPR5/OLD1,* cell death

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. Currently, two major theories of evolutionary senescence are widely acknowledged (Kirkwood and Austad, 2000). The Antagonistic Pleiotropy Theory points out that evolution acts to maximise the reproduction fitness and will allow the existence of genes that have beneficial effects for early-life survival and reproduction despite the fact that these genes 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 mutations with deleterious late-life effects will allow the carrier to reproduce before dying and lead to senescence. These two theories implicate that two classes of genes are responsible for senescence: genes 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 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; Gem and Patridge, 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 program that leads to the death of plant cells, tissues, organs, and whole plants. Leaves are a model system for plant senescence studies. There is a debate 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 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). Due to such a strong adaptive advantage, leaf senescence appears to violate the definition of evolutionary senescence that occurs at 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 (Bleecker, 1998). In fact, at biochemical and molecular levels, leaf senescence resembles animal ageing in various aspects. It has been argued that in most cases, the strategies used by plants to regulate senescence are similar to those in animals (Gan, 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 defense-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 the 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.

In *Arabidopsis*, several senescence regulatory genes have been identified through mutational analyses (Gan, 2003; Lim et al., 2003). The *cpr5/hys1* alleles were isolated in a screen for mutants with altered dark-induced whole plant senescence (Yoshida et al., 2002a). They were also recovered in screens for mutants with constitutive expresser of pathogenesis related (*PR*) genes (Bowling et al., 1997) or abnormal trichome development (Kirik et al., 2001) suggesting that leaf senescence may share common regulatory components with pathogen resistance and cell proliferation. Besides the aforementioned phenotypes, *cpr5* mutants showed an elevated salicylic acid (SA) level, enhanced sugar sensitivity, reduced plant size, and spontaneous lesion formation (Bowling et al., 1997; Yoshida et al., 2002a). *CPR5* encodes a plant-specific protein that has two distinct signatures: a nuclear localization signal and at least four transmembrane domains (Kirik et al., 2001; Yoshida et al., 2002a). The stunted stature of *cpr5* mutants indicates that *CPR5* acts to ensure normal growth and development at early life. The abnormal early development of *cpr5* mutants may relate to the elevated SA and the enhanced sugar sensitivity since both SA and sugar are able to regulate cell growth in *Arabidopsis* (Vanacker et al., 2001; Rolland et al., 2002). In addition, the prominent senescence-associated and lesion mimic cell death processes may contribute to the compromised growth and development.

The accelerated leaf senescence in *cpr5* mutants might be a consequence of constitutive activation of pathogen resistance responses. Plant disease resistance responses-and senescence have several intrinsic associations. Both processes are genetically controlled and require the active participation of the cell for its own diminishing (Pennell and Lamb, 1997; Dangl and Jones, 2001; Lam et al., 2001; Lim et al., 2003). *SAGs* were found to express in cells near the sites of pathogen challenge (Pontier et al., 2000), whereas *PR* genes were shown to be up-regulated during leaf senescence as well (Buchanan-Wollaston, 1997; Quirino et al., 1999;

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	$120^{G\ to\ D}$
cpr5-1	Col-0	GGT to GAT	$420^{G\ to\ D}$
cpr5-2	Col-0	TGG to TAG	$473^{W\ to\ stop}$
hys1-1	Col-0	TGG to TGA	$477^{W\ to\ stop}$

Buchanan-Wollaston et al., 2003). A genetic hierarchy elucidating the role of *CPR5* in mediating a broad spectrum of disease resistance has been constructed, in which *CPR5* was placed upstream of salicylic acid (SA), ethylene (ET), and jasmonic acid (JA) signalling pathways (Bowling et al., 1997; Clarke et al., 2000; Jirage et al., 2001). Thus, it is possible that the accelerated senescence is caused by the alteration in these hormonal signals, which have been demonstrated to be important senescence modulators (Grbic and Bleecker, 1995; Morris et al., 2000; He et al., 2001).

In an effort to isolate *Arabidopsis* mutants that exhibit altered ethylene-induced leaf senescence, we obtained *old1* mutants, which caught our special attention for two distinct alterations: accelerated age-regulated senescence and enhanced ethylene responses (Jing et al., 2002). *old1* was found to be allelic to *cpr5*, and hence in the *cpr5*-induced phenotypes, two more items were added. More importantly, *old1* mutants differed from previous reported *cpr5* mutants in that they showed predominantly senescence but no macroscopic lesions. We took advantage of this genetic variation and the available hormonal mutants to dissect the diverse functions of *CPR5* and to explore the possible roles of the affected signalling pathways in *cpr5*-induced phenotypes. Transgenic plants with increased *CPR5* mRNA levels were also constructed to examine the effects of ectopic expression of *CPR5* on leaf senescence. We showed that *CPR5* mediated multiple signals to control seedling growth and to repress cell death in adult plants. However, *CPR5* can promote leaf senescence at 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.

Results

Identification of L*er*-0 alleles of *cpr5*

The *old1* mutants were isolated in a screen for leaf senescence mutants that showed accelerated visible yellowing upon exposure to 10µl/l ethylene for 3 days; the details of this screen were reported previously (Jing et al., 2002). Map-based cloning showed that *old1* alleles were allelic to *cpr5* and hence were renamed as *cpr5-l1*, *cpr5-l2* and *cpr5-l3*, respectively (Table 1). These L*er*-0 *cpr5* mutants showed phenotypes different from *cpr5* or *hys1* mutants derived from Col-0 (see below). We took the advantage of the available *cpr5* alleles to examine the roles of *CPR5* in various aspects of *Arabidopsis* development, especially its role in senescence regulation.

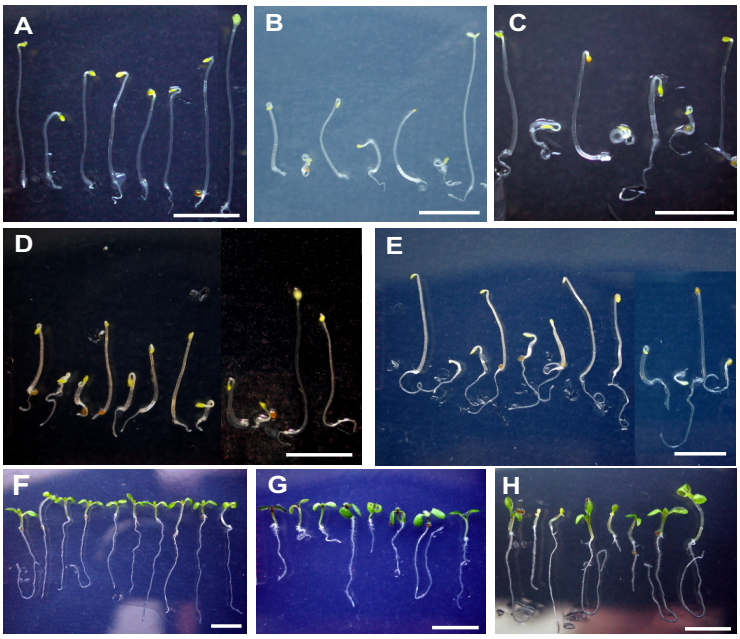

Figure 1. Enhanced responses of *cpr5* mutant and double mutants to various signalling molecules.
(**A-C**). 5-d-old seedlings grown in darkness on MS (**A**), MS supplemented with 1µM ACC (**B**) or 0.5% sucrose (**C**). The first 6 seedlings are L*er*-0, *cpr5-l1*, Col-0, *cpr5-2, hys1-1, cpr5-l1/C*, and to the right are *abi4-1* and *ein2-1* (**A**), *ein2-1* (**B**), and *abi4-1* (**C**).
(**D-E**). 7-d-old seedlings grown in darkness on MS supplemented with 10µM ACC (**D**), or 1% sucrose (**E**). Seedlings were in the same order for the two figures and from left to right are: L*er*-0, *cpr5 l1*, *cpr5-l1/C,* Col-0, *cpr5 2, hys1-1, abi4-1, cpr5-l1abi4-1, ctr1-1, cpr5-l1ctr1-1, ein2-1,* and *cpr5-l1ein2-1.*
(**F-H**). 7-d-old seedlings grown under light on MS and 0.5% sucrose (**F**), MS and 0.5% sucrose supplemented with 20µM MeJA (**G**), or MS and 0.3µM ABA (**H**). The first 6 seedlings are L*er*-0, *cpr5-l1*, *cpr5-l1/C,* Col-0, *cpr5 2, hys1-1,* and to the right are *jar1-1, cpr5-l1jar1-1, abi4-1*and *cpr5-l1abi4-1* (**F**), *jar1-1* and *cpr5-l1jar1-1* (**G**), and *abi4-1* and *cpr5-l1abi4-1* (**H**).
The experiments were repeated at least three times with similar results and representative seedlings are shown. White bars represent 0.5cm.

Enhanced responses of *cpr5* mutants to ET, sugar, JA and ABA

Two prominent phenotypes of *cpr5* mutants were the enhanced responses to sugar and ET (Jing et al., 2002; Yoshida et al., 2002a). Several *cpr5* mutants are in the Col-0 background while the *cpr5-l1* mutants have the L*er* background. The *cpr5-l1* mutant was crossed to Col-0 and several F2 plants, homozygous for the *cpr5-l1* allele, were selected and designated as *cpr5-l1/C*. These mutants have a Col-0 and L*er* mixed background and allow for a better comparison between the *cpr5* alleles from the different genetic backgrounds. Original exams showed that the individual *cpr5-l1/C* alleles performed similar in a variety of assays. Therefore, one line was selected for further study. Figure 1 shows the observed variation among *cpr5* alleles in their enhanced responses to ET and sugar. In the ET triple response assay, *cpr5-l1* and *cpr5-l1/C* seedlings exhibited the strongest hypersensitivity and the seedlings were ring-shaped. While the *cpr5-2* mutants displayed modest shortening in the hypocotyl length,

no obvious differences between *hys1-1* mutants and the wild types were observed (Figure 1B). Similar allelic differences were observed in the sugar sensitivity assay (Figure 1C). Interestingly, the allelic variations appear to correlate with the positions of the mutations (Table 1), the closer the defect is to the C-terminal end of the protein, the less pronounced the response. To further examine the relationship between these two signalling pathways in *cpr5* mutants, double mutants between *cpr5-l1* and *ctr1-1, ein2* and *abi4* were constructed and their ET and sugar sensitivity examined (Figure 1D-E). These hormonal mutants were isolated from the Col-0 background, thus the responses of the double mutants were compared with *cpr5-l1/C.* The results showed that *ctr1-1* and *ein2* mutations exaggerated or blocked the enhanced ET response of *cpr5-l1,* respectively. However, the *ein2* mutation did not affect the sugar hypersensitivity. On the other hand, *abi4* alleviated the exaggerated sugar response of *cpr5*, but not its ET hypersensitivity. These results suggested that the enhanced ET and sugar sensitivities are independent downstream events after the *cpr5* mutation.

The *cpr5* mutations also resulted in hypersensitivity to JA and ABA (Figures 1F-H). The enhanced JA response of *cpr5-l1* was restored by *jar1*, and ABA sensitivity by *abi4*, suggesting that *CPR5* acts upstream of *JAR1* and *ABI4*.

Taken together, *cpr5* mutants exhibit enhanced responses to ET, sugar, JA, and ABA, indicating that in seedlings *CPR5* may function by controlling multiple signalling pathways.

Elevated JA levels in *cpr5* mutants

The altered hormone responses may be a result of changed hormone levels. Therefore, the levels of several hormones were measured in the *cpr5* mutants and wild types. Figure 2 shows the endogenous levels of SA and JA in rosette leaves 3 and 4 of 21-d-old soil-grown *cpr5-l1* mutants and various double mutants. Consistent with the analysis of other *cpr5* mutants (Clarke et al., 1997; Clarke et al., 2000; Clarke et al., 2001; Jirage et al., 2001), the *cpr5* mutants showed a similar elevated endogenous SA level (data only shown for *cpr5-l1/C* mutant). The SA level was substantially reduced by the bacterial *nahG* gene or increased by the *npr1* mutation (Figure 2A). Interestingly, *ein2* did not affect the SA level in *cpr5-l1*. This effect of *ein2* was different from previously reported in another *cpr5* mutant in which *ein2* was shown to increase the SA level 3 fold (Clarke et al., 2000). However, *ctr1-1* reduced the *cpr5-l1* SA level, suggesting that constitutively activating ET signalling had a negative effect on SA production. Similarly, *jar1* was found to repress the SA production in *cpr5-l1* as envisaged by the lower level of SA in *cpr5-l1jar1*.

The JA level was also increased in the *cpr5* single mutants (Figure 2B, data only shown for *cpr5-l1/C*), which may be the cause for the observed hypersensitivity to JA. In *cpr5-l1nahG* and *cpr5-l1npr1* plants, the JA level was ~5 and ~2 fold as high as in *cpr5-l1* mutants, respectively. *ein2* increased JA level in *cpr5-l1* mutants, but *ctr1-1* did not affect the JA level. *jar1* had a slightly but significantly higher JA level than the wild types and it substantially increased the JA level in *cpr5-l1*. We also measured the ET production in etiolated *cpr5-l1* mutants and its various double mutants and the results showed that *cpr5-l1* had a wild-type ET production level, and that SA and JA seemed not to affect ET production (data not shown).

Thus, *cpr5* mutants possessed not only elevated SA levels, but also high JA levels. *ctr1* and *jar1* reduced the high level of SA in *cpr5-l1,* while *ein2, nahG* and *jar1* increased JA levels in *cpr5-l1.*

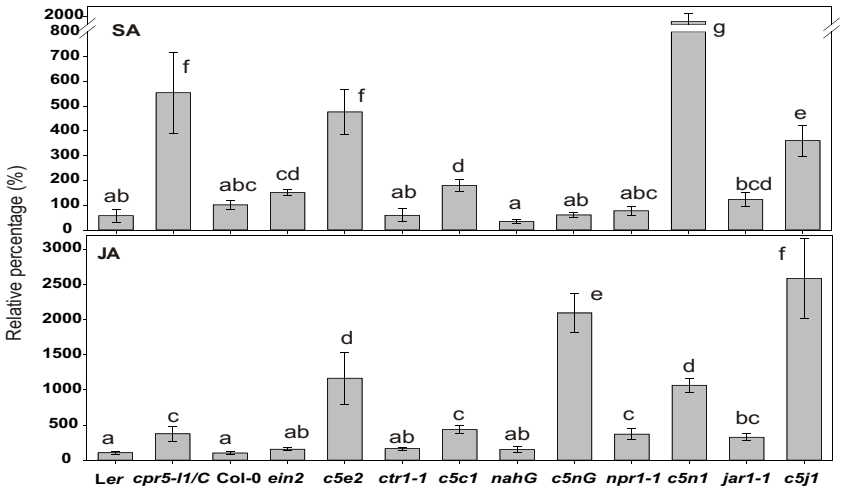

Figure 2. Relative endogenous SA and JA levels in wild type and mutant plants.
Rosette leaves 3 and 4 from 21-d-old soil-grown seedlings were used for HPLC-MS quantification as detailed in Experimental procedures. Results are presented as relative percentage of the content of Col-0. Lines with the same letters are not significantly different from each other at the α=0.05 significant level as determined by Ducan Multiple Variant Mean Test. The absolute values for Col-0 were 75±12 (mean±sd) ng/g fresh weight for SA and 6.7±0.5 ng/g fresh weight for JA. For each line, at least 4 replicates were used for quantification. *c5e2* stands for *cpr5-l1ein2; c5c1* for *cpr5-l1ctr1-1; c5nG* for *cpr5-l1nahG; c5n1* for *cpr5 l1npr1-1;* and *c5j1* for *cpr5-l1jar1-1*.

The senescence syndrome in *cpr5* mutants and double mutants

The effects of hormones in *cpr5*-induced senescence were characterised using a genetic approach. We examined the senescence syndrome using a combination of visible, physiological and molecular markers. A distinction was made between whole leaf yellowing and the occurrence of chlorotic lesions. Representative plants were photographed after 30 days of growth in air and are shown in Figure 3. Leaf yellowing was quantified by measuring average chlorophyll contents in cotyledons and the first and second true leaf pair separately (Figure 4). The senescence syndrome was further examined by determining the expression profiles of several senescence and cell death marker genes as shown in Figure 5. We included a reliable developmental senescence marker *SAG12*, a general cell death marker *SAG13*, and the pathogenesis-related gene *PR-1* (Lohman et al., 1994; Bowling et al., 1997; Weaver et al., 1998; Miller et al., 1999; Noh and Amasino, 1999; Brodersen et al., 2002).

The wild type plants and the single hormonal mutants did not show signs of senescence under our growth conditions, whereas the *cpr5* mutants showed clear visible yellowing (Figure 3, data shown for the *cpr5* mutants). As opposed to *cpr5-l1*, in which the visible yellowing appeared uniformly, *cpr5-l1/C* and *cpr5-2* also showed a mixture of yellow patches and randomly spreading chlorotic lesions. Moreover, in rosette leaves 3 and 4 the chlorophyll content of *cpr5-l1* was lower than those of the other two mutants (Figure 4). These results indicate that the genetic background affects the pattern of visible yellowing caused by *cpr5* alleles. Despite the differences in the visible yellowing, *cpr5-l1*, *cpr5-l1/C* and *cpr5-2* exhibited

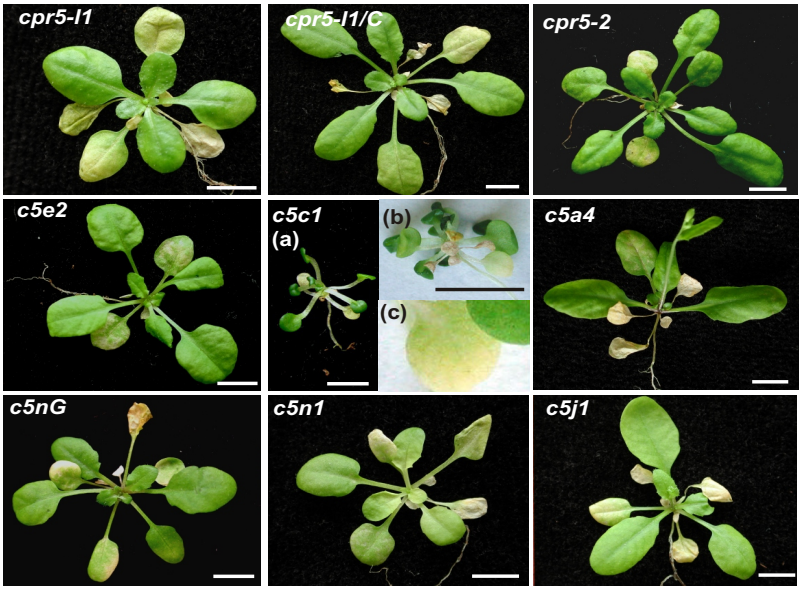

Figure 3. Senescence phenotypes of *cpr5* mutants and double mutants.
Plants were grown on soil under the conditions described in Experimental procedures for 30 days and representative plants were selected and photographed. All plants had a similar amount of rosette leaves except *c5c1* plants that grew slower than others as envisaged by the fewer numbers of leaves (**a**). A representative 35-d-old *c5c1* plant with similar leaf numbers was taken and photographed upside down (**b**). An enlarged view of a *c5c1* rosette leaf was shown (**c**). The abbreviations are the same as in Figure 2. Bars represent 1cm.

very similar *SAG* expression and all the senescence marker genes *SAG12*, *SAG13*, *SAG14*, and *SAG21* showed higher expression levels than those observed in wild type. *PR-1* mRNA level was also higher in *cpr5* mutants (Figure 5).

The ET signalling mutations *ein2* and *ctr1* had opposite effects on the senescence syndrome in rosette leaves. In *cpr5-l1ein2* only prominent chlorotic lesions were observed, whereas in *cpr5-l1ctr1-1*, the whole leaf blades were uniformly yellow and no macroscopic lesions were observed (Figure 3). Trypan blue staining and subsequent observation of microscopic lesions revealed that the *cpr5* mutants showed microscopic lesions, comparable to those that were observed in 45-day-old senescing L*er*-0 and Col-0 leaves (data not shown). In *cpr5-l1ein2* the observed microscopic lesions corresponded to the macroscopic lesions, whereas in c*pr5-l1ctr1-1* no microscopic lesions were observed. Compared with *cpr5-l1/C*, the rosette leaves 3 and 4 of *cpr5-l1ein2* showed less pronounced yellowing and higher chlorophyll content, whereas *cpr5-l1ctr1-1* showed more visible yellowing and lower chlorophyll content. Gene expression was similarly differentially affected by the two ET signalling mutations. The *SAG12* mRNA level of *cpr5-l1ein2* was not detectable, whereas in *cpr5-l1ctr1-1*, it was increased to a higher level than that of *cpr5-l1/C*. In contrast, the *SAG13* mRNA level in *cpr5-l1ein2* was comparable to that of *cpr5-l1/C*, whereas it was not detected in *cpr5-l1ctr1*. The *SAG14* and *PR-1* mRNA levels in *cpr5-l1* were not affected by *ein2*, but were reduced by *ctr1-1*. Compared to *cpr5-l1/C*, the *SAG21* mRNA level was reduced in *cpr5-l1ein2*, but was similar in *cpr5-l1ctr1-1*.

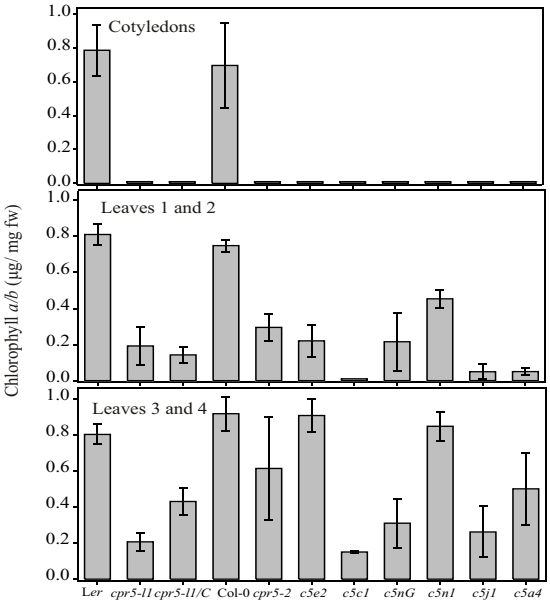

Figure 4. Comparison of chlorophyll contents between the wild type, *cpr5* mutants and double mutants.
Plants were grown on soil under the conditions described in Experimental procedures for 30 days (*c5c1* plants for 35 days) and leaf pairs were sampled for chlorophyll content measurement. Results are shown as mean±sd of at least 4 replicates. The abbreviations are the same as in Figure 2 and *c5a4* stands for *cpr5-l1abi4-1*.

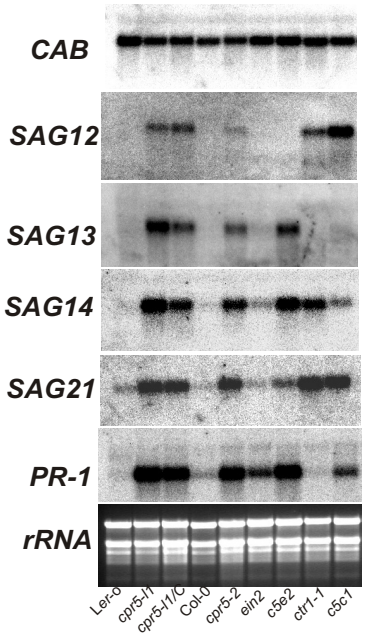

Figure 5. Northern hybridisation analysis showing the abundance of the mRNA of various genes in wild type plants, *cpr5* mutants and double mutants. Total RNA from the whole rosette of 30-d-old plants (35-d-old *c5c1* plants) was isolated and 10µg RNA samples were used. ^{32}P labelled cDNA probes were used. An rRNA picture is shown as a loading control. The abbreviations are the same as in Figure 2.

The bacterial *nahG* gene and the *npr1* mutation were shown to reduce the senescence syndrome in *Arabidopsis* (Morris et al., 2000). The senescence syndrome in *cpr5-l1npr1* was weaker than in *cpr5-l1/C*. For instance, the yellowing in *cpr5-l1npr1* did not cover the whole leaf blades and the chlorophyll contents in the examined rosette leaves were higher than those in the *cpr5-l1/C* leaves (Figures 3, 4). In *cpr5-l1nahG* plants the yellowing was more

Figure 6. The senescence syndrome of *Arabidopsis cpr5-1* mutants and transgenic lines with different *CPR5* mRNA levels.

(**A**). Comparison of *CPR5* mRNA levels of *Arabidopsis* transgenic lines, the *cpr5-1* mutant, and the wild type. The relative abundance of *CPR5* to ubiquitin (*UBQ5*) mRNA, expressed as mean±sd is shown.

(**B, C**). 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 (**B**) and second (**C**) 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 days and collected for chlorophyll content measurement. Four replicates of 2 pairs of leaves were analyzed for each line. Results are shown as mean±sd. Plant lines with the same letters are not significantly different from each other at the α=0.05 significant level as determined by the Ducan Multiple Variant Mean Test.

(**D, E**). Comparison of *in planta* senescence of *CPR5* transgenic lines, the *cpr5-1* mutant and the wild type. Plants were grown on soil for 50 days and the numbers of yellow leaves were recorded as described in Experimental procedures. **D** shows representative plants whose inflorescence stems were removed. White bars in **D** represent 1cm. **E** shows the results of visible yellowing quantification that are presented as mean±sd from observations on at least 10 plants per line.

(**F**). Northern hybridization 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 total RNA was used for Northern blotting analysis. ^{32}P labelled probes were used. An rRNA picture is shown as a loading control.

lesion-like, as compared to *cpr5-l1/C*, but there was no difference in chlorophyll content. Introducing *nahG* or *npr1* into *cpr5-l1* could reduce the mRNA levels of *SAG12* and *PR-1* but did not affect those of *SAG13, SAG14* and *SAG21* (data not shown). JA, ABA and sugar were shown to regulate *SAG* expression (He et al., 2002; Rolland et al., 2002). However, we did not observe obvious differences among *cpr5-l1/C*, *cpr5-l1jar1* and *cpr5-l1abi4*.

Thus, enhancing ET signalling hastened the senescence syndrome but inhibited chlorotic lesion formation in *cpr5* mutants, whereas impaired ET signalling led to reduced senescence symptoms and increased lesion formation. The *nahG* transgene and *npr1* mutation could suppress some aspects of the senescence syndrome in *cpr5* mutants.

Accelerated senescence in *CPR5* overexpression lines

Transgenic plants that express varied amounts of *CPR5* transcripts were constructed to examine the effects of *CPR5* expression on leaf senescence. The *CPR5* mRNA levels in leaves from 21-d-old soil-grown plants were measured with a light cycler and the results showed that lines *C5-7, C4-3* and *N5-6* had higher than wild type *CPR5* mRNA levels, line *C8-6* and *cpr5-1* mutants had lower levels (Figure 6A). Figure 7 shows the phenotypes of the silenced line *C8-6*, which displayed all the *cpr5*-mutant phenotypes such as the enhanced hormonal sensitivity, reduced leaf and plant size and abnormal trichome development. These altered phenotypes were not observed in the overexpression lines.

We examined senescence phenotypes in the transgenic lines. Figures 6B and 6C show the results of the detachment-induced senescence experiment. As could be expected, *cpr5-1* mutants 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 *cpr5-1* mutants and *C8-6* plants. Similar results

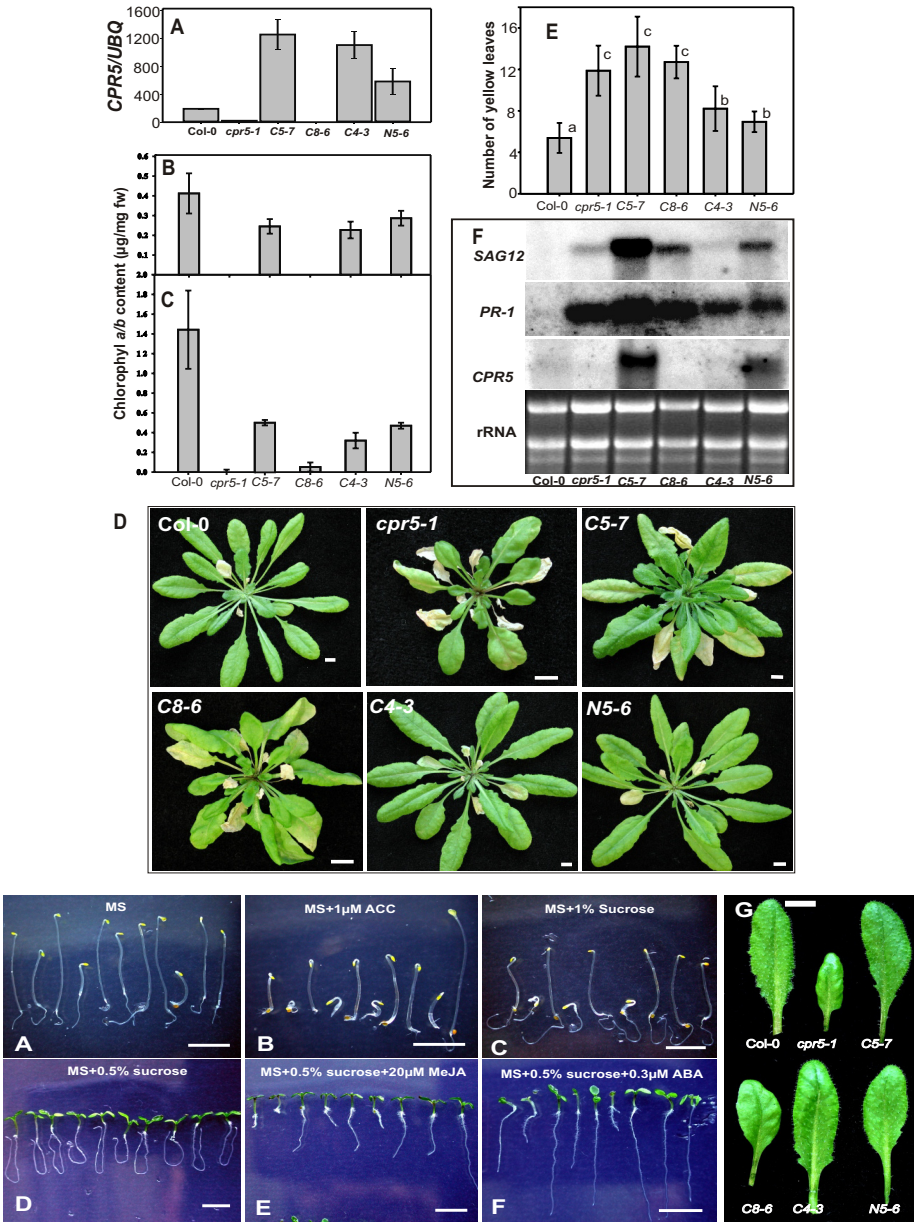

Figure 7. Characterisation of hormonal sensitivity and growth of *CPR5* transgenic lines, *cpr5* mutants and wild type.

(**A-F**) The first 8 seedlings are L*er*-0, *cpr5-1*, 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 indicated components, in darkness for 5 days (**A-C**) or in light for 7 days (**D-F**). (**G**) Representative mature leaves from the indicated lines. White bars represent 0.5cm.

were obtained when the detached leaves were incubated with JA or ABA (data not shown). Thus, the leaves of the transgenic lines exhibited accelerated drops in chlorophyll content upon detachment. We further characterised the developmental senescence. Similar to *cpr5* mutants, the *C8-6* plants displayed visible yellowing at young plant stages and at the time of bolting yellow cotyledons and at least two yellow rosette leaves were observed (data not shown). The *CPR5*-overexpressing lines were indistinguishable from the wild type up to the bolting stage (approximately 40 days after germination). At ~40 days after germination visible yellowing was observed both in the wild type and the transgenic lines. In the overexpression lines, however, visible yellowing proceeded faster and occurred in younger leaves. Figures 6D and 6E show that at 50 days after germination similar numbers of yellow leaves were observed in *cpr5-1* mutants and lines *C8-6* and *C5-7*, and that these numbers were significantly higher than those observed in wild type plants. Lines *C4-3* and *N5-6* also showed significantly more yellowing than the wild type. Thus, visible yellowing was accelerated in the transgenic plants. The correlation between *CPR5* expression and the advanced senescence syndrome was further studied using molecular markers (Figure 6F). Northern blotting detection showed that at this developmental stage, the *CPR5* mRNA level was the highest in *C5-7,* slightly lower in *N5-6* and comparable with the wild type in *C4-3*. This was a surprising finding, since *CPR5* mRNA levels were very high in 21 day-old *C4-3* plants. Nevertheless, there was a good correlation between the *CPR5* and *SAG12* mRNA levels. Very low *CPR5* levels (*cpr5-1* and *C8-6*) as well as higher than wild type *CPR5* levels (*C5-7* and *N5-6*), correlated with increased *SAG12* mRNA levels. The increased visible yellowing and *SAG12* mRNA levels in *C4-3* plants may be the result of the higher *CPR5* expression levels during earlier development. *PR-1* mRNA levels, furthermore, correlated well with the visible yellowing.

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

CPR5 differentially mediates multiple signalling pathways

The *cpr5* mutants have many phenotypes and were recovered in several mutant screens, indicative of its involvement in various aspects of the plant life cycle. On the basis of the results presented here and those obtained from several groups, we propose that *CPR5* functions to repress a common signalling integration complex as shown in Figure 8. We examined the responses of *cpr5* seedlings to various signalling molecules at different developmental stages.

Seedlings carrying *cpr5* alleles were found to exhibit enhanced ET and sugar responses (Jing et al., 2002; Yoshida et al., 2002a) and were hypersensitive to JA and ABA. These signalling molecules all contribute to seedling growth and development in various ways (Creelman and Mullet, 1997; Johnson and Ecker, 1998; Rolland et al., 2002). It has been documented that complex cross-talks exist among ET, sugar and ABA signalling pathways (reviewed by Rolland et al., 2002; Cheng et al., 2003; Leon and Sheen, 2003). For instance, *ein2* mutants are insensitive to ET but hypersensitive to ABA and sugar, whereas *ctr1* mutants exhibit constitutive ET response but are ABA and sugar insensitive (Guzman and Ecker, 1990; Zhou et al., 1998; Beaudoin et al., 2000). These results suggest that ET signalling acts antagonistically with sugar and/or ABA signalling. However, we did not observe similar

interactions in *cpr5* seedlings, under our experimental conditions. Epistatic analyses indicated that compromising sugar signalling by introducing *abi4*, did not affect ET hypersensitivity, and *vice versa*. Furthermore, we observed a mutation-position specific variation in the sensitivity to ET and sugar among different alleles, which was not observed in JA or ABA hypersensitivity. Thus, the enhanced hormonal responses appeared to be partially parallel and independent events in *cpr5* seedlings.

In adult *cpr5* plants, we observed complex interactions in the control of the endogenous levels of ET, SA and JA among the *cpr5* mutants, as outlined in Figure 8. Previous studies have shown that a high level of SA suppresses the accumulation of JA (Peña-Cortés et al., 1993; Doherty et al., 1988) and that the JA levels increase after pathogen infection only in the absence of SA accumulation (Spoel et al., 2003). It has also been shown that *cpr5* mutations result in elevated SA levels (Bowling et al., 1997). Here we found that in the *cpr5* mutant, the JA level was also increased, suggesting that both biosynthetic pathways can be activated simultaneously. Nevertheless, *nahG* or *npr1* strongly increased the JA levels in *cpr5-1l*, confirming that SA has an antagonistic effect on JA production. On the other hand, JA signalling might also act antagonistically to control the SA level since *jar1* decreased the SA level in *cpr5*. ET signalling appeared to influence the SA and JA levels in *cpr5*. Interestingly, this effect was unidirectional, with only constitutive ET signalling repressing SA and JA since we did not observe differences in the ET production among *cpr5* alleles and various double mutants.

Two prominent phenotypes of adult *cpr5* mutants are the abnormal trichome development and reduced plant size. Both phenotypes were not affected in all of the examined double mutants, suggesting that SA, ET, ABA and JA signalling components are not involved in these abnormalities.

Taken together, we conclude that *CPR5* differentially mediates multiple hormonal signals depending on the plant developmental stage.

CPR5 controls different cell death processes

CPR5 is a repressor of cell death and two different processes were readily visible in *cpr5* plants: senescence-associated and lesion-mimic. The senescence-like cell death, however, could be a result of massive lesion formation. Similarly, the lesion formation could be the result of localized senescence. Making use of double mutants between *cpr5* and hormonal pathway mutants in combination with molecular markers, we provided several lines of evidence that *cpr5* controls the two cell death processes independently.

First, at the macroscopic level, the L*er*-0 *cpr5* mutants showed predominantly accelerated senescence and lacked chlorotic lesions, whereas lesions were evident in *cpr5* mutants in Col-0 or Col-0 and L*er*-0 mixed background. Thus, the genetic background changes the appearance of the two cell death processes. More importantly, they involved different signalling pathways: the senescence phenotype of *cpr5-1l/C* mutants depended on the ethylene signalling pathway, while the lesion mimic phenotype did not. Introducing *ein2* into *cpr5-1l* reduced the senescence symptoms and caused a reduction of the expression of the natural senescence marker *SAG12* (Lohman et al., 1994; Weaver et al., 1998; Miller et al., 1999; Noh and Amasino, 1999), to undetectable levels. In contrast, neither chlorotic lesion formation nor the mRNA levels of the general cell death markers *SAG13* and *PR-1* (Uknes et al., 1992; Brodersen et al., 2002) were affected. In a complementary experiment, *ctr1-1* was introduced into *cpr5-1l* mutants. In *cpr5-1l/ctr1-1* there was a concomitant increase in *SAG12* expression

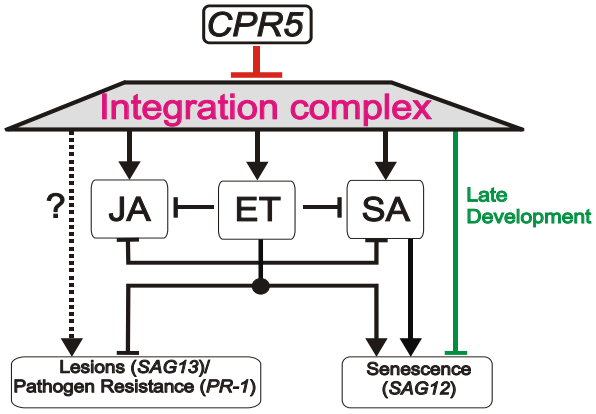

Figure 8. A tentative model depicting the functions of *CPR5* in regulating cell death via mediating multiple signalling pathways. *CPR5* is proposed to function through an integration complex and employ ET, SA and an undefined signalling pathway (dotted line) to regulate leaf senescence and lesion formation. The promotive effect of *CPR5* on senescence is shown as a green T-bar. The interactions among ET, SA and JA are also shown. Arrows indicate promotion and T-bars stand for inhibition. See discussion for details.

and senescence symptoms in younger leaves. In the same plants, *SAG13* and *PR-1* mRNA levels were reduced and lesions were absent. Thus, in *cpr5* mutants, ET signalling promoted leaf senescence but inhibited lesion formation. Such functions of ET in *cpr5*-induced cell death differ from its role in pathogen resistance-associated cell death where ET was shown to be a stimulator of lesion expansion after pathogen attack (Bent et al., 1992; Lund et al., 1998; Pilloff et al., 2002). Other hormone signalling pathways had a varied effect on senescence symptoms and lesion formation. Reducing SA levels or blocking SA signalling reduced *SAG12* and *PR-1* mRNA levels, but had no effect on lesion formation in *cpr5* mutants (Bowling et al., 1997), suggesting that *CPR5* might regulate additional signals to control lesion formation (Figure 8). Remarkably, our results showed that inhibiting JA responses by *jar1* affected neither lesion formation nor senescence, even though the role of JA in both senescence and defence has been well documented (Penninckx et al., 1998; Pieterse et al., 1998; He et al., 2002). Thus, the precocious cell death in *cpr5* mutants may not involve JA signalling (Figure 8). Finally, in *CPR5*-overexpressing plants, early leaf senescence was uncoupled from lesion formation and a correlation was found between *CPR5* and *SAG12* expression levels. *PR-1* mRNA levels, however, did not correlate with *CPR5* mRNA levels in all transgenic lines. Taken together, these observations indicated that distinct cell death processes occurred in *cpr5* mutants as outlined in Figure 8.

The discussion presented above argues that mutations in *CPR5* resulted in accelerated cell death including early leaf senescence and hence indicates that *CPR5* functions to repress senescence. However, *CPR5* overexpression lines exhibited enhanced leaf senescence both upon detachment and *in planta*, as envisaged by the earlier appearance of visible yellowing, faster drop of chlorophyll content, and a correlation of the *SAG12* mRNA levels with the *CPR5* mRNA levels. The promoting effect of *CPR5* on senescence was obtained by ectopic expression, which might not reflect the real function of *CPR5* in the wild type. Nonetheless, accelerated leaf senescence was the only unique phenotype in *CPR5* overexpression lines.

Furthermore, the expression pattern of CPR5 was examined in transgenic plants harbouring *CPR5* promotor:GUS reporter constructs and the results indicated that CPR5 levels increased in mature leaves (unpublished results), which is consistent with the reports that a roughly 2-fold increase in *CPR5* transcript was found in senescing leaves (Zimmermann et al., 2004; https://www.genevestigator.ethz.ch.). Finally, ectopic expression was employed to demonstrate the regulatory function of another senescence regulatory gene *SAG101* (He and Gan, 2002). Thus, it seems likely that the accelerated leaf senescence obtained from *CPR5* overexpression indeed reflects the promotion effect of *CPR5* in wild type plants.

Together, analyses on *cpr5* mutants show that *CPR5* is required to repress cell death, while overexpression analyses show that *CPR5* can promote senescence-associated cell death. A plausible explanation for these seemingly contradicting results is that *CPR5* may display changed functions during development. Indeed, the senescence syndrome in *cpr5* mutants or transgenic plants with low *CPR5* mRNA levels was distinctively different from the one in *CPR5* overexpression lines. In the former ones, senescence started early in adult plants, whereas in *CPR5* overexpression lines it started late in life after bolting. Thus, at adulthood, *CPR5* was required to repress leaf senescence and another cell death process, whereas at late development it could promote senescence (Figure 8).

Evolution of senescence regulating genes in plants

Besides leaf senescence, *CPR5* is also involved in seedling growth, trichome development, and pathogen resistance, indicative of its pleiotropic nature. A common feature of several senescence regulatory genes is that they are involved in plant growth and development as well. *ORE9* encodes an F-box protein and is part of the ubiquitination protein degradation machinery (Woo et al., 2000). *ore9/max2* alleles were also recovered in screen for mutants with altered shoot lateral branching (Stirnberg et al., 2002). Furthermore, *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). This fits very well to the prediction of the antagonistic pleiotropy theory of senescence (Kirkwood and Austad, 2000). Moreover, these observations agree with the view that similar molecular strategies appear to have evolved for senescence regulation in both plant and animal kingdoms (Gan, 2003; Lim et al., 2003).

This notion might be particularly true for *CPR5*. 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. Interestingly, *CPR5* also shows high expression in embryos (https://www.genevestigator.ethz.ch.). However, at late developmental stage, a functional *CPR5* promotes "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. The insulin/IGF-1 signalling pathway has pleiotropic functions and is shown to control lifespan, reproduction and stress resistance in many organisms (Guarente and Kenyon, 2000; Gems and Partridge, 2001; Kenyon, 2001; Tatar et al., 2003). A recent study in nematode worms indicates that *DAF-2,* an insulin/IGF-1-like receptor, employs independent mechanisms to regulate lifespan and reproduction, and that the insulin/IGF-1-like pathway influences development and reproduction at early life, but acts exclusively

during adulthood to control adult lifespan (Dillin et al., 2002). p53 is a genome guardian, the deficiency of p53 proteins leads to cancer and tumor development due to increased cellular damages, 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 tumors than wild-type ($p53^{+/+}$) homozygotes, but exhibited faster ageing (Tyner et al., 2002). Clearly, maintaining a higher p53 level at 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.

Experimental procedures

Plant material 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 as *cpr5-l1* in this paper) (Jing et al., 2002), *cpr5-1* (Bowling et al., 1997), *cpr5-2* (Boch et al., 1998), *hys1-1* (Yoshida et al., 2002a), *npr1-1* (Cao et al., 1994), *ein2-1* (Guzman and Ecker, 1990), *ctr1-1* (Kieber et al., 1993), *jar1-1* (Staswick et al., 1992), *abi4-1* (Finkelstein et al., 1998), and *Arabidopsis* plants expressing the bacterial *nahG* gene (Bowling et al., 1994). Depending on the specific phenotypes and identities of the particular mutations, a variety of screening methods employing hormonal responses and PCR-based marker assays were used to isolate double mutants.

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 centiMorgans 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 generate by crossing *cpr5-l1* to 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 programme, 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 (Table1). *Agrobacterium*-mediated transformation was performed to further confirm 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, mutant *cpr5-1* (lacking *NptII*) and *cpr5-2* plants (Clough and Bent, 1998). Transformants were selected on MS media containing 50 µg/mL kanamycin (Murashige and Skoog, 1962) and complementation of *cpr5* was determined by restored wild-type trichome development and lack of macroscopic lesions. Lines homozygous for single insertion events were used in further experiments.

Hormonal sensitivity assay

For ET sensitivity, seedlings were grown on MS media containing 1µM ACC (1-aminocyclopropane-1-carboxylic acid) in the dark for 5 days, and the triple response was observed (Guzman and Ecker, 1990). Sugar sensitivity was determined by growth for 5 days 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 20µM MeJA, and the root elongation of 7-d 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 0.3µM ABA. The growth of 7-d light-grown seedlings was examined.

Chlorophyll content measurement and gene expression analysis

For chlorophyll content and Northern blotting, rosette leaf samples were collected from 30-day-old soil-grown Arabidopsis plants and were prepared and analyzed as described by Jing et al. (2002).

For light cycler 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. (1994). Ten µg of RNA was treated with DNase I according to manufacturer's instructions (Ambion Inc., Austin, Texas). One µg of RNA was incubated with Superscript II reverse transcriptase and oligo dT in a 20µl reaction to synthesize cDNA (Invitrogen, Carlsbad, California). For the quantitative PCR half reactions (10µl each), 2µl of the cDNA product was used as template with the *CPR5* specific primers, whereas 2µl of a 20-fold dilution was used for reactions with *Ubiquitin5* (*UBQ5, At3g62250*) specific primers. The final primer concentration in all reactions was 0.5µM. Quantitative PCR was carried out using the SYBR green PCR kit (QIAgen, Valencia, California) 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 3 replicate experiments by normalization to *UBQ* transcript levels.

HPLC-MS (High Pressure Liquid Chromatograph-Mass Spectrometry) analyses of salicylic acid and jasmonic acid

Rosette leaves number 3 and 4 without any signs of visible yellowing were taken from 21-day-old soil-grown plants and used to measure the SA and JA concentrations according to a procedure derived from Wilbert et al. (1998). Briefly, ~200mg leaf tissues were ground in liquid

nitrogen into fine powder and extracted with 500µl 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 100µl 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 adding 1% NH_4OH solution in MeOH with a flow of 100µl/min, 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 2.1mm-column (Alltech Alltima C18 5µ). The gradient was delivered by 2 Perkin-Elmer series 200 LC-pumps at a flow rate of 200µl/min, started with 30% MeOH for 1min, raised to 95% in 5min and retained for 5min, then dropped to 30% in 2min. 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 to 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 200µl/min HPLC-flow, combined with a 100µl/min. 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 136-139amu and 92-95amu with a step-size of 0.100amu and a dwell-time of 15ms was analyzed. 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 208-211amu with the same step-size and dwell-time was analyzed. The molecular weight of JA is 210. The M-1-ion is m/z 209. We avoided SIM to double-check the isotope-patterns of the free acids. Due to the interference of many unknown products, slightly 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 into the plant extracts as controls.

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Chapter 5
Arabidopsis onset of leaf death 3 **encodes a mutated cytosolic *O*-acetylserine (thiol) lyase that results in enhanced cadmium tolerance and early leaf senescence**

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Abstract
The *Arabidopsis onset of leaf death (old) 3* is a co-dominant mutation that causes seedling-lethal, early senescence symptoms. The phenotype segregated as a monogenic trait in the parental line L*er*-0 and 4 other accessions but as a two-gene controlled trait in Col-0, Ws-0 and Wil-2. This resulted from the specific interaction between *old3* and a L*er*-0 gene, named *ODD* (for *old3 determinant*). Our data implied that the corresponding Col-0 *odd* allele was either different or not present at all. Map-based cloning showed that *old3-1* causes a gly^{162} to glu^{162} substitution in the cytosolic *O*-acetylserine (thiol) lyase (OAS-TL). Leaf senescence and sulphur metabolism was examined in *old3-1ODD* mutants, in *old3-1odd* mutants with a L*er*-0 and Col-0 mixed background but with the *odd* allele from the Col-0 accession, and in a Col-0 *old3-Todd* mutant containing a T-DNA insertion in the OAS-TL gene. Leaf senescence was only enhanced in *old3-1ODD* mutants. Altered sulphur balances (the contents of the total, ionic and organic sulphur) and lower thiol levels were observed in *old3-1ODD* and *old3-Todd* mutants, but not in *old3-1odd* mutants. Thus, the *old3-1* allele requires the *ODD* gene for the altered sulphur metabolism and early leaf senescence phenotypes. Cadmium tolerance was enhanced in *old3-1ODD* and *old3-1odd* mutants but reduced in *old3-Todd* mutants, showing that the *ODD* gene is not required for the enhanced cadmium tolerance phenotype. Together, the *old3* OAS-TL gene has novel features of enhancing cadmium tolerance and leaf senescence.

Keywords: *Arabidopsis*, cadmium tolerance, cysteine synthesis, leaf senescence, *old3*, *O*-acetylserine thiol lyase (OAS-TL), sulphur metabolism

Introduction
Sulphur is essential for life and plants play an important role in the biological sulphur cycle. Cysteine is the building block for sulphur-containing organic components including glutathione that have been implicated in the adaptation of plants to a wide range of biotic and abiotic stresses (May et al., 1998). Cysteine synthesis creates a link between sulphur reduction and amino acid metabolism and therefore is a point of convergence for nitrogen and sulphur assimilation.

Sulphur assimilation starts with the transportation of anionic sulphate into plant cells by a gene family of plasma membrane associated proton/sulphate co-transporters (Buchner et al., 2004). Through serial enzymatic reactions, sulphate is converted into sulphide, which, together with *O*-acetylserine (OAS), forms cysteine in a reaction catalysed by *O*-acetylserine (thiol) lyase (OAS-TL) (Leustek et al., 2000; Saito, 2000). OAS is synthesised from serine and acetyl-CoA and this reaction is catalysed by sulphur acetyltransferase (SAT). Detailed biochemical studies have shown that OAS-TL and SAT physically interact with each other and can form an enzymatic complex or dissociate from each other depending on the status of sulphur, OAS and cysteine inside the cell (Saito, 2000; Hell et al., 2002; Noji and Saito, 2002). SAT is only active when it is associated with OAS-TL, whereas OAS-TL is only active when it is dissociated from SAT (Hell et al., 2002). The SAT to OAS-TL protein expression ratio suggests that SAT is the limiting factor for the reaction (Wirtz et al., 2004). In a number of species, both OAS-TL and SAT were shown to have three subcellular isoforms in cytosol, chloroplasts and mitochondria, but their contribution to the overall cysteine biosynthesis is unclear. Interestingly, the cytosolic SAT/OAS-TL complex is subject to cysteine feedback control and

is postulated to sense the sulphur nutrition status and fine-tune the overall cysteine synthesis in plants (Hell et al., 2002; Noji and Saito, 2002).

Organisms are not programmed for senescence or death but are programmed for survival (Kirkwood and Austad, 2000). It has been recognised that plant general metabolic and developmental processes are important for senescence regulation (Woo et al., 2002; Gan, 2003; Jing et al., 2003). In *Arabidopsis*, several senescence regulation genes have been identified through mutational analyses and were shown to have general functions in plant growth and development (Woo et al., 2000; He et al., 2002; Woo et al., 2003). Our study showed that *CPR5/OLD1* might be a senescence regulation gene with multiple functions throughout plant development (Chapter 4). Cysteine-derived glutathione and other secondary organic sulphur components are involved in the scavenging of free radicals and hence are implicated in the oxidative damage induced cell death processes including leaf senescence (Navabpour et al., 2003). Nonetheless, little molecular genetic evidence is available to show a causal link between leaf senescence and cysteine biosynthesis or sulphur metabolism in general.

We studied the interaction between leaf age and ethylene and isolated several classes of *onset of leaf death* (*old*) mutants with altered leaf senescence (Jing et al., 2002). Among the mutants isolated, a single *old3-1* mutant line was isolated from the Landsberg *erecta* (L*er*-0) accession. To elucidate the molecular basis of *old3*-induced early leaf senescence, cloning of *old3* was initiated and revealed that *old3-1* results in an amino acid substitution in the cytosolic *O*-acetylserine (thiol) lyase. The cysteine synthesis, sulphur balance and cadmium tolerance were examined in *old3* mutants and their relationships with *old3*-induced senescence discussed.

Results

Arabidopsis accessions varied in the control of *old3*-induced phenotypes

A single *old3-1* allele was isolated from an EMS-mutagenised *Arabidopsis* L*er*-0 population in a screen for mutants with altered ethylene-induced senescence. *old3-1* was shown to be a co-dominant trait (Figure 1A; Jing et al., 2002). The senescence syndrome of *old3-1* occurred in air-grown plants but was not further enhanced by exogenously applied ethylene. A rapid drop in cotyledon chlorophyll content occurred in *old3-1* between 12-14 days after germination, and was followed by an increase in ion leakage (Figure 1B). Similar changes were not observed in wild type plants during this period. When *old3-1* mutants were grown in a light intensity of 250$\mu mols^{-1}cm^{-2}$, the lethal phenotype of homozygous *old3-1old3-1* plants were rescued and the early senescence phenotypes of heterozygous *old3-1OLD3* plants delayed (Figure 1C). This implies that the early senescence phenotype of *old3* mutants is not the result of a general growth defect.

Plants heterozygous for the *old3-1* mutations were crossed to Col-0 and all the F_1 progeny showed the wild type phenotype, instead of a 1: 1 ratio of wild-type: mutant in L*er*-0 (Table 1; Jing et al., 2002). The segregation pattern of F_2 progeny also deviated from 1: 2: 1 of wild-type: intermediate: mutant. These results revealed that Col-0 and L*er*-0 differ in the control of *old3-1* phenotypes. Initial mapping showed that *old3-1* phenotypes were linked to SSLP marker K11J14 on chromosome 3 and to CAPS marker G4539a on chromosome 4 (Jing et al., 2002). Thus, in addition to *old3-1*, a second gene is involved in the control of *old3* phenotypes. The gene that determines the *old3* phenotype was designated as *ODD* (*old3 determinant*). The L*er*-0 allele was called *ODD* and the Col-0 allele *odd*. *old3-1ODD* was crossed to *fca-1* and *abi1-1* (both having the L*er* background) to clarify which of the loci represents *old3*. *old3-1*

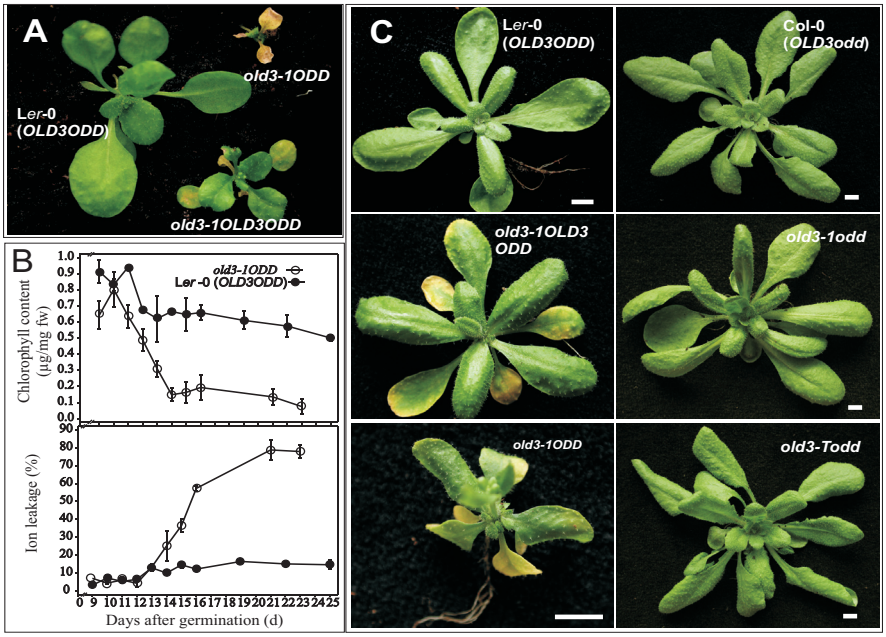

Figure 1. Phenotypes, chlorophyll content and ion leakage of *old3-1* mutant lines and wild types.
(**A**). Representative 21-d-old soil-grown plants of L*er*-0, heterozygous (*old3-1OLD3ODD*) and homozygous (*old3-1ODD*) mutants, grown in 60µmolcm^{-2}s^{-1} cool white fluorescent light.
(**B**). Chlorophyll content and ion leakage of cotyledons from L*er*-0 plants and *old3* mutants grown in 60µmolcm^{-2}s^{-1} cool white fluorescent light. Each data point is shown as mean ± sd deviation from 4 replicates.
(**C**). Representative 27-d-old soil-grown plants of L*er*-0, heterozygous (*old3-1OLD3ODD*) and homozygous (*old3-1ODD*) mutants, Col-0, *old3-1odd*, and *old3-Todd*, grown in 250µmolcm ^{2}s^{-1} cool white fluorescent light. Bars represent 0.5cm.

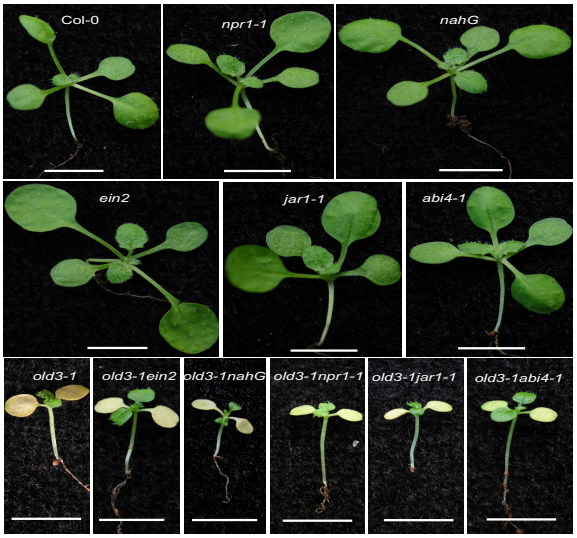

Figure 2. Phenotypes of *old3* and its various double mutants in comparison with wild type, hormonal mutants and the nahG transgenic plant.
Double mutants were isolated as described in Experimental procedures and were homozygous for both the *old3-1* and the *ODD* gene. Plants were grown on soil in 60µmolcm^{-2}s^{-1} cool white fluorescent light for 17 days and representative plants were photographed. Bars repreesent 0.5cm.

Table 1. The genotypes, phenotypes and segregation of F_1 and F_2 progeny of the *old3* heterozygously crossed to the parental lines Ler-0 and several other *Arabidopsis* accessions[1]

Male	Female	F_1			F_2			PCR marker
		Genotype	Phenotype	WT: mutant	Genotype	Phenotype	WT:He:Ho	
old3-1 OLD3 ODD ODD	Bu-18, Di-2, L*er*-0, Wa-1, Rsch-0,	*OLD3OLD3 ODDODD* *old3-1OLD3 ODDODD*	Wild type He-*old3*	1:1	*OLD3OLD3oddodd* *old3OLD3oddodd* *old3old3oddodd*	Wild type He-*old3* Ho-*old3*	1:2:1	10kb
old3-1 OLD3 ODD ODD	[2]Ak-1, Bd-0, Bla-2, Bs-2, Col-0, Litva, Mt-0, Nok-0, Rubezhnoe-1, Tsu-1, Wil-2, Ws-0	*OLD3OLD3 ODDodd* *old3-1OLD3 ODDodd*	Wild type Wild type	1:0	ODDODDOLD3OLD3 old3 OLD3ODDODD old3old3ODDODD *OLD3OLD3ODDodd* *old3OLD3ODDodd* *old3old3ODDodd* *OLD3OLD3oddodd* *old3OLD3oddodd* *old3oldoddodd3*	Wild type Wild type Wild type Wild type Wild type He-*old3* Wild type He-*old3* Ho-*old3*	11:4:1	2.5kb

[1]: The pollen from He-*old3* plants was used to individually pollinate various *Arabidopsis* accessions indicated in the female column. The possible genotypes and corresponding phenotypes of the resultant F_1 progeny are shown. In the crosses between He-*old3* and L*er*-0 and its similar accessions, only the F_1 progeny with the mutant phenotypes was selected and continued for F_2 segregation analyses. In the crosses between He-*old3* and Col-0 and its similar accessions, at least 10 F_1 plants were selected and allowed to set seed and the phenotypes of F_2 populations were observed. The segregation analyses were done with at least 500 plants in the F_2 populations containing the mutant phenotypes. The PCR marker was examined in the female *Arabidopsis* accessions as described in Experimental procedures.

[2]: Among these accessions similarly containing the 2.5kb PCR fragments, Col-0, Wil-2 and Ws-0 were crossed to He-*old3* for genetic segregation analyses.

was found to co-segregate with *abi1-1* and *fca-1* (data not shown). Thus, *old3-1* is located on chromosome 4 and *ODD* on chromosome 3.

old3 is a downstream regulator of leaf senescence

old3 was proposed to work later in a senescence regulatory pathway (Jing et al., 2002). To further understand the position of *old3* in the senescence regulatory network, we performed a detailed epistatic analysis to clarify the roles of several senescence-promoting hormones in *old3*-induced senescence. *old3-1ODD* mutants from the L*er*-0 background were crossed to *ein2*, *npr1-1, abi4-1, jar1* mutants, or transgenic plants harbouring the *NahG* construct. All these mutants have the Col-0 background and thus contained the *odd* allele. Mutants were isolated that were homozygous for the *old3-1*, *ODD* and hormonal pathway mutations, using molecular markers as described in Experimental procedures. As shown in Figure 2, impairing ethylene, salicylic acid, jasmonic acid, abscisic acid, or sugar signalling, did not change the

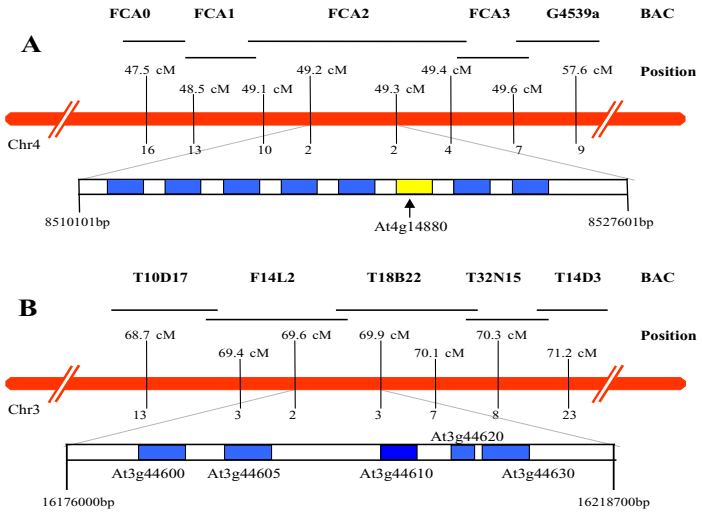

Figure 3. A diagram showing map-based cloning of *old3-1* and *ODD*.
The BACs spanning the mapped regions are shown. The tested SNP markers are shown by their positions on the Lister & Dean RI map and on the physical map. The number of recombination events between *old3-1* (A) or *ODD* (B) and a particular marker is shown below the marker. The *OLD3* gene and the predicted genes in the *ODD* region are indicated.

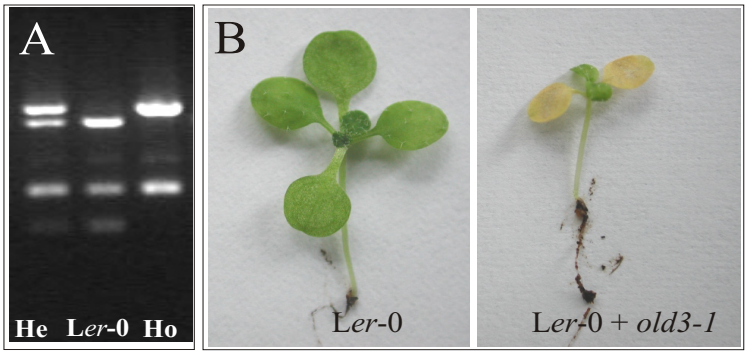

Figure 4. Molecular characterisation of the *old3* mutation.
(A). An agarose gel photograph showing the polymorphism between wild type L*er*-0 gene and *old3-1ODD* at the nucleotide sequence level. PCR-products were amplified from L*er*-0, *old3-1ODD* (Ho) and *old3-1OLD3ODD* (He) mutants and restricted with *Sau96I*.
(B). Generation of *old3-1* phenotypes in L*er*-0 wild type plants. The genomic sequence spanning the *old3-1* region was cloned and transformed to wild type as described in Experimental procedures. A representative 14-day old L*er*-0 plant and a L*er*-0 plant transformed with the *old3-1* gene are shown.

seedling-lethal phenotype of *old3-1* plants. Although the double mutants could survive and finish the whole life cycle under an elevated light intensity similar to the single *old3-1* mutant, they showed early leaf senescence (data not shown). These results suggest that the early senescence phenotype of *old3-1ODD* mutants does not depend on these hormonal pathways.

***OLD3* encodes the cytosolic *O*-acetylserine (thiol) lyase (OAS-TL)**

Further mapping placed *old3-1* into a 14-kb region on bacterial artificial chromosome clone FCA2 (accession number Z97337) spanning 8 open reading frames (Figure 3A). The *old3-1* genome of this region was sequenced and a GGA to GAA codon change was found at the fifth exon of the gene encoding the cytosolic *O*-acetylserine (thiol) lyase (OAS-TL, *AT4G14880*). The *old3-1* mutation generated a Sau96I restriction polymorphism between *old3-1* and the wild type (Figure 4A). At the protein level, it changes a glycine at position 162 to glutamic acid. The amino acid sequences of the OAS-TL isoforms are highly conserved among plant species and share very high homologies to those in unicellular organisms (e.g. the wild type OAS-TL shares 49% identities with that of *Salmonella typhimurium*, for a review, see Leustek et al., 2000). According to the crystallised structure of the enzyme from *S. typhimurium*, the substitution caused by *old3-1* is located at the sixth α-helix of the encoded protein (Burkhard et al., 1998).

A complementation test was conducted to further confirm the isolation of *old3-1*. Because of the co-dominant nature, the mutated *old3-1* genomic sequence was cloned and transformed into wild-type L*er*-0 plants. The transformants were found to display the same phenotypes as the *old3-1* mutants (Figure 4B). Thus, the product of the mutated *OAS-TL* gene is responsible for the phenotypes generated by *old3-1*.

***old3* works in pair with the L*er*-0 *ODD* allele to induce early leaf senescence**

Our initial genetic evidence indicated that the L*er*-0 and Col-0 *ODD/odd* alleles function different in *old3*-induced early leaf senescence. Using a mapping population, the gene was fine mapped to a 52-kb region containing 5 predicted open reading frames on chromosome 3 spanning parts of the bacterial artificial chromosome clones F14L2 and T18B22 (Figure 3B). We took advantage of the availability of the genomic sequences of TAIR Col-0 and Cereon L*er*-0 databases and performed *in silico* comparison of the five predicted genes. No differences were found in the coding sequence of *AT3G44600*, which is predicted to have high similarity to *homo sapiens* cyclophilins. *AT3G44605* has a high similarity with a retroelement transposon fragment and was therefore not believed to encode ODD. The remaining region in the Col-0 genome sequences contains three predicted open reading frames encoding a protein kinase (*AT3G44610*), a phosphotyrosine protein phosphatase (*AT3G44620*), and a putative TIR-NBS-LRR class disease resistance protein (*AT3G44630*), respectively. However, large variation was found in this area between Col-0 and L*er*-0 genome and no direct genic synteny was possible. A 10-kb L*er*-specific PCR fragment could be amplified from the region between *AT3G44610* and *AT3G44620.* When Col-0 DNA was used as a template, a 2.5 kb fragment was amplified, consistent with the genome sequence. The polymorphism for this marker was further examined in additional 6 accessions and the results showed that these accessions split into two groups, either containing the 10-kb fragment as in L*er*-0, or the 2.5-kb fragment as in Col-0 (Table 1). The *old3-1OLD3ODD* mutants were crossed to several accessions, and the results showed that in the accessions containing the 10-kb fragment (Di-2, Bu-18, Rsch-0 and Wa-1), the *old3-1* phenotypes behaved as in L*er*-0, but in those (Ws-0 and Wil-2) containing the 2.5-kb fragment behaved as in Col-0. Sequencing of the 10-kb fragment did not reveal an intact open reading frame.

The co-dominant nature of the *odd* allele suggested that, similar to *old3-1*, the activity of the L*er ODD* gene, rather than the absence of the Col-0 *odd* allele, is required for the

manifestation of the *old3-1* phenotypes. To validate this hypothesis, a L*er*-0 binary BAC library (Chang et al., 2003) was probed with the 10-kb fragment as described in Experimental procedures. *old3-1odd* plants (containing *old3-1* allele in a Col-0 and L*er*-0 mixed background and the Col-0 *odd* allele) harbouring the positive BAC clones exhibited *old3-1ODD* phenotypes (data not shown). Thus, the L*er*-0 *ODD* allele is essential and the specific interaction between *old3-1* and the *ODD* gene is responsible for the observed early leaf senescence phenotype.

Sulphur metabolism and physiology is altered in *old3* mutants

The OAS-TL gene family works at the last step of sulphur assimilation and synthesis of cysteine is the key step for the formation of other organic sulphur-containing compounds (Saito, 2000). We examined sulphur-associated physiology in *old3-1* mutants (Figure 5). Two more mutant lines were included for a better comparison. One SALK knock-out line containing a T-DNA insertion inside the *OAS-TL* gene was isolated as described in Experimental procedures and designated as *old3-Todd*. In an F_2 population generated from an *old3-1OLD3ODD* x Col-0 (*OLD3OLD3odd*) cross, plants with *old3-1odd* genotypes were selected. Observations on the identified *old3-1odd* lines showed that they performed similarly in a number of growth conditions and hence only one line was used for further study. Under our growth conditions, differences in leaf senescence were not observed among *old3-Todd*, *old3-1odd* and wild type plants (Figure 1C). This further confirmed that the *ODD* allele is required for the early leaf senescence phenotype.

As shown in Figure 5A, the *old3-1ODD* and *old3-1OLD3ODD* mutants in the L*er*-0 background exhibited reduced average dry weight as compared to wild type, concomitant with the early onset of leaf senescence. *old3-1odd* plants did not differ from Col-0 in the average dry weight, but a higher average dry weight was found in *old3-Todd*. The contents of total, ion and organic sulphur are direct measurements of plant sulphur balance and hence were quantified in the mutants and wild type plants. In *old3-Todd*, the total sulphur content was increased as compared to Col-0. This increase was associated with an increase in the ion sulphur contents. Compared to L*er*-0, *old3-1OLD3ODD* and *old3-1ODD* exhibited higher contents of total and ion sulphur, but *old3-1odd* did not differ from L*er*-0 or Col-0. The organic sulphur contents could be measured by the ratio between the total and ion sulphur contents. All the examined lines had a similar level of organic sulphur. Among plant organic sulphur compounds, the water-soluble non-protein thiols are related to various stress responses (Rauser, 1993; Cobbert, 2000; Gotor et al., 2003) and therefore were examined in the mutants. *old3-Todd* contained a lower thiol content than Col-0. In comparison with L*er*-0, the thiol content was lower in *old3-1ODD*, but higher in *old3-1OLD3ODD*. *old3-1odd* displayed a thiol content similar to Col-0, but lower than L*er*-0. Thus, the *old3* mutation caused different effects on the total sulphur, ion sulphur and thiols contents.

We further performed two bioassays related to sulphur physiology. Cysteine can be added into the culture media as an instant organic sulphur source in plant growth assays (e.g. Herschbach and Rennenberg, 1995; Dominguez-Solis et al., 2001). Hence, the *old3-1ODD* mutant seedlings were grown in cysteine-containing media to examine whether cysteine feeding could alleviate the *old3-1* mutant phenotype. Figure 5B shows that the mutant phenotypes did not change, indicating that cysteine deficiency was not the cause of the *old3-1* phenotypes.

The cytosolic OAS-TL has been shown to be important for cadmium tolerance in *Arabidopsis* (Dominguez-Solis et al., 2001), we tested the growth of the *old3* mutant lines

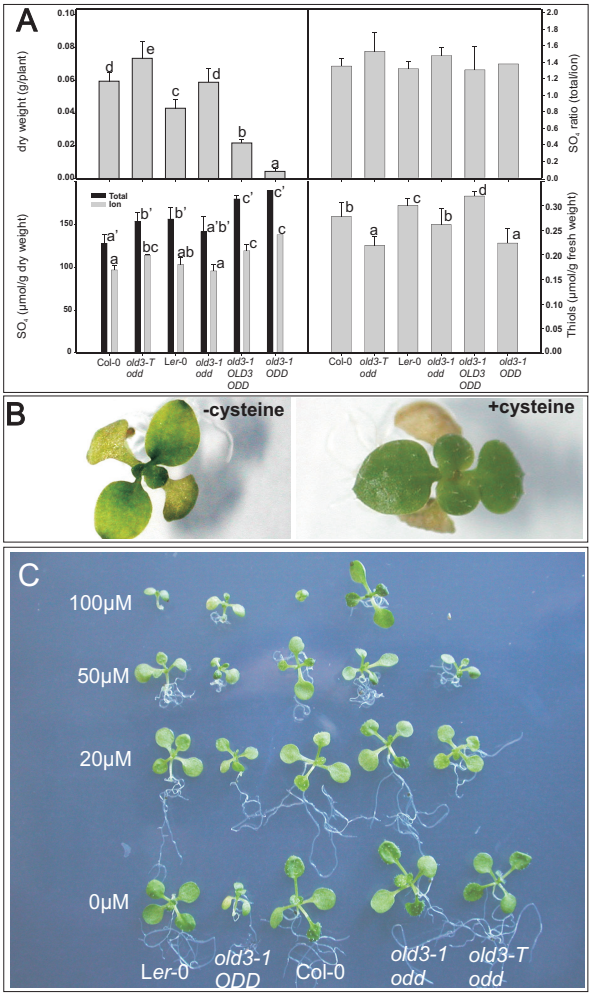

Figure 5. Characterisation of sulphur-associated physiology in *old3* mutants. (A). Dry weight accumulation, total, anion and organic sulphate content, and thiol content of wild types and *old3* mutant lines. Plants were grown for 27-days and the aboveground parts were harvested for analysis as described in Experimental procedures. Data are shown as mean ± standard deviation of four replicates. One replicate of 50 plants was used for the analysis of the total and anion sulphate content of homozygous *old3-1ODD* mutants. Bars with the same letters are not significantly different from each other after Ducan multiple variant significance test at a significance level of 0.05.
(B). Phenotypes of 24-d-old *old3-1ODD* mutants grown on MS medium and 1% sucrose with and without 250µM cysteine.
(C). Phenotypes of L*er*, *old3-1ODD,* Col-0,, *old3-1odd* and *old3-Todd* plants, grown for 17 days on MS medium and 1% sucrose in the presence of the indicated concentrations of cadmium.

together with wild type plants on various cadmium concentrations (Figure 5C). No differences were observed among all the tested lines when the cadmium chloride concentration was 0 to 20µm, except that the *old3-1ODD* seedlings were smaller and showed signs of visible yellowing on cotyledons. At 50µm $CdCl_2$, a reduced growth occurred. Clear differences were observed at 100µM $CdCl_2$. Under this condition, *old3-Todd* seeds showed only the protrusion of radicles and post-germination growth was completely inhibited. The development of wild type seedlings was arrested at the cotyledon stage. However, both *old3-1ODD* and *old3-1odd* were still able to grow showing elongated roots and growth of the first pairs of rosette leaves. Interestingly, early senescence was still evident in *old3-1ODD* plants. Thus, *old3-Todd* is hypersensitive to cadmium ions but *old3-1ODD* and *old3-1odd* mutants exhibit enhanced tolerance.

Taken together, these results indicated that the *old3-1* together with the *ODD* causes an altered sulphur balance. In contrast, *old3-T* shows an altered sulphur balance, independent of *ODD*. Further, *old3-T* causes a reduced cadmium tolerance, while the *old3-1* mutation enhances cadmium tolerance independently of the *ODD* mutation.

Discussion

In this study, the previously isolated *old3-1* mutant was characterised. *old3*-induced leaf senescence is controlled by two genetic loci, *old3* and *ODD*. Our epistatic analyses of double mutants between *old3-1* and mutants with a defect in ET, SA, JA, or ABA/sugar signalling suggested that *old3*-induced leaf senescence does not involve these senescence signalling molecules. Thus, *old3* may act downstream in the senescence regulatory network as previously proposed (Jing et al., 2002). Map-based cloning showed that *old3-1* caused an amino acid substitution in the cytosolic OAS-TL, an enzyme implicated in cysteine synthesis. The results generated here allowed us to dissect the genetic basis of *old3*-induced leaf senescence and to exploit a causal link between regulation of leaf senescence and a general metabolic process.

Genetic basis for *old3*-induced early leaf senescence

Two genes are involved in the regulation of *old3*-induced leaf senescence. Apart from *old3-1*, a second gene *ODD* is required, which is present in the L*er* genetic background. Among 8 accessions tested, 5 contain an *ODD* allele, with similar properties as the L*er*-0 *ODD* allele, while 3 contain an *odd* allele, similar to the one found in the Col-0 accession. Interestingly, the presence of *odd/ODD* alleles does not correlate with the geographic origins of the accessions, or with their phylogenic relationships as illustrated with molecular markers (Barth et al., 2002).

The results show that *old3-1* encodes a functional gene as transgenic presence of the *old3-1* allele in otherwise wild type L*er* plants causes the *old3-1* phenotype. Similarly, the activity of the L*er ODD* allele is required for the *old3-1* senescence phenotype. Thus, the senescence phenotype depends on the presence of both the *old3-1* allele and the L*er ODD* gene, implying that the two genes may directly interact. At present, the identity of the *ODD* gene is unknown. Although *ODD* is mapped to a region encompassing *AT3G44610* and *AT3G44620* in Col-0, we were unable to pinpoint the gene since the genome sequence is highly polymorphic between the L*er*-0 and Col-0 accessions in this part of the genome. A recent thorough experimental analysis of transcriptional activity in Col-0 genome has identified over 3000 new genes (Yamada et al., 2003). Thus, *in silico* prediction of genes will not detect all genes and *odd* may not be an annotated gene yet. Our data showed the situation may be even more complex when functional genes are compared among different accessions. Indeed, the Col-0 *odd* allele is either different or not present since leaf senescence is manifested only when both *old3-1* and *ODD* are present. At the moment, the *ODD* gene allele was mapped to a 52-kb region and further molecular analysis is required to reveal the true identity of the *ODD* gene.

Plants showing *old3-1* phenotypes were homozygous for both *old3-1* and *ODD* alleles. Those with intermediate phenotypes contained either two copies of *old3-1* and one copy of *ODD*, or two copies of *ODD* and one copy of *old3-1*. Thus, to generate early senescence phenotype, a specific interaction between *old3-1* and *ODD* appears to be required and their effects are gene-dosage dependent. Such a pattern resembles the naturally occurring ecotype-specific interactions between *FLOWERING LOCUS D (FLD)* and *FLOWERING LOCUS C (FLC)* in the control of flowering time, although the proposed *old3-ODD* interaction is generated by mutagenesis. *fld* is a recessive allele causing delayed flowering, which segregated in a typical 1:3 ratio in Col-0, but in Ler-0 *fld* generates minimal phenotype, suggesting the involvement of another Col-0 gene in the control of *fld*-induced delayed flowering (Sanda and

Amasino, 1996). The *fld*-interacting gene turned out to be *FLC*, which is truncated in L*er*-0 due to the insertion of a nonautonomous *Mutator*-like transposon in the first intron that is required for normal *FLC* regulation (Gazzani et al., 2003). Thus, such ecotype-specific interaction may well be preserved in senescence regulation as well.

The relationship between sulphur metabolism and leaf senescence in *old3* mutants

Plants carrying a mutation in the *old3* gene showed a changed sulphur metabolism and physiology, including altered sulphur balance, changed thiol levels, and cadmium tolerance. However, *old3-1ODD*, *old3-1OLD3ODD*, *old3-Todd* and *old3-1odd* mutants displayed different alterations. The sulphur balance as monitored by the total, ionic and organic sulphur content, was altered in *old3-1ODD, old3-1OLD3ODD* and *old3-Todd*, but was not changed in *old3-1odd* mutants; the thiols were lower in *old3-1ODD*, higher in *old3-1OLD3ODD*, but not changed in *old3-1odd*. These results show that in addition to the *old3-1*, the *ODD* gene is required to cause alterations in sulphur metabolism. Thus, in *old3* mutants, early leaf senescence occurs concomitant with alterations in sulphur metabolism.

At present it is not clear what is the causal relationship between the senescence phenotype and the alterations in sulphur metabolism. It is possible that early leaf senescence is the cause of the altered sulphur metabolism. During senescence, ionic sulphate contents are expected to increase due to the remobilisation of nutrients (Himelblau and Amasino, 2001), and this could be partially the reason why the ionic sulphate contents increased in *old3-1ODD* and *old3-1OLD3ODD*. Furthermore, it has been shown that in *Arabidopsis* leaves, the thiol levels initially increased at the onset of senescence, but decreased at late senescence (de Kok and Graham, 1989). *old3-1OLD3ODD* mutants exhibited less obvious senescence symptoms and a higher than wild-type thiol level. Therefore, the thiol levels in *old3-1OLD3ODD* and *old3-1ODD* may mimic the changes of thiol levels in wild type plants during senescence. On the other hand, the altered sulphur metabolism can be the cause of the accelerated leaf senescence in *old3* mutants. Sulphur depletion results in up-regulation of genes involved in jasmonic acid biosynthesis and genes induced by jasmonic acid or methyl jasmonic acid (Hirai et al., 2003; Maruyama-Nakashita et al., 2003; Nikiforova et al., 2003). Changed sulphur nutrition may affect senescence through the action of jasmonic acid, whose role in leaf senescence has been substantiated (He et al., 2002). The expression of nitrilase, genes which are involved in auxin biosynthesis, were up-regulated and free IAA levels were increased upon sulphur starvation (Hirai et al., 2003; Nikiforova et al., 2003). Similar changes were reported during senescence (Quirino et al., 1999). Recently, sulphurtransferases have been found to be one type of senescence-associated genes (Meyer et al., 2003). These studies provide circumstantial evidence that sulphur alteration may affect leaf senescence. However, the effect of the changes in sulphur balance on leaf senescence in the *old3-1ODD* mutants remains unclear.

Several lines of evidence suggest that the *old3-1* mutation may not result in the complete elimination of OAS-TL activity. First, the senescence symptoms were not changed when *old3-1* mutants were grown in the presence of exogenously applied cysteine. Second, in contrast to the effect of the *old3-T* mutation, the *old3-1* mutation did not affect sulphur metabolism in the absence of *ODD*. Finally, the *old3-T* and *old3-1* mutation have opposite effects on cadmium tolerance. Clearly, the mutation caused by the T-DNA insertion has a different effect than the amino acid substitution caused by *old3-1*, suggesting that *old3-1* may encode a functional OAS-TL.

Intriguingly, the enhanced cadmium tolerance in plants containing the *old3-1* allele did not depend on *ODD*: cadmium tolerance was similarly enhanced in *old3-1ODD* and *old3-1odd* mutants, while tolerance was reduced in *old3-Todd* mutants. The molecular mechanism of the enhanced cadmium tolerance is unclear. Plant thiols are predominantly present in the form of glutathione, which is implicated in plant responses to various stresses including heavy metal toxicity (Cobbert, 2000). Cysteine is the precursor for glutathione synthesis and the pools of the non-protein thiols might be important for cadmium tolerance. However, no correlation has been found between the thiol level and the cadmium tolerance in the various *old3* mutants. Consistently, it has been shown that the absolute levels of thiols do not correlate with freezing tolerance in wheat (Stuiver et al., 1995). *De novo* synthesis of cysteine and gluthathione was proposed to be essential for the coupling reaction with cadmium (Dominguez-Solis et al., 2001). Significant increases in the mRNA level and the enzymatic activity of the cytosolic OAS-TL have been observed within 1 hour after cadmium treatment, and transgenic plants overexpressing the cytosolic OAS-TL display enhanced tolerance to cadmium. Therefore, it is possible that the *old3* mutation may cause an enhanced OAS-TL activity allowing a rapid response upon cadmium stress. The three-dimensional structure of the *Salmonella typhimurium* OAS-TL has been resolved (Burkhard et al., 1998). The proposed OAS-binding pocket includes four essential residues, Ser^{69}, Gly^{70}, Asn^{71}, and Thr^{72}, which are conserved in all the plant OAS-TL characterised up to date. Plant OAS-TLs are expected to have a structure that is similar to the one from *Salmonella*, owing to the high sequence similarity. The Gly^{162} to Glu^{162} substitution in the *old3-1* mutated OAS-TL is located at the sixth α-helix and is outside the substrate-binding site. Thus, it is possible that the *old3-1* OAS-TL may have a changed three-dimensional conformation with an increased cysteine synthetic activity. Alternatively, the Arabidopsis cytosolic OAS-TL has been shown to play a central regulatory role (Saito, 1999; Leustek et al., 2000; Hell et al., 2002) and the *old3-1* mutation may enhance the overall control on cysteine biosynthesis and/or cysteine coupling with cadmium. No matter what the exact molecular mechanism are, our results show that *old3-T* and *old3-1* mutations appear to have different effects on the cytosolic OAS-TL activity as envisaged by their effects on cadmium tolerance.

In summary, the cloning and characterisation of *old3-1* revealed that *OLD3* encodes a cytosolic OAS-TL and that the *old3-1* gene is functional with novel features of enhancing cadmium tolerance. Furthermore, *old3-1* discloses a novel link between sulphur metabolism and leaf senescence in combination with the *ODD* gene. Future cloning of the *ODD* gene and characterisation of its molecular interaction with *old3-1* will allow the regulation of leaf senescence to be viewed from a fresh perspective.

Experimental procedures

Plant material and growth conditions

Arabidopsis thaliana accessions L*er*-0 and Col-0 were the wild types. The Arabidopsis accessions Bu-18, Di-2, Wa-1, Rsch-0, Wil-2 and Ws-0 were obtained from the NASC. The mutant alleles and transgenic plants used were *old3-1* (Jing et al., 2002), *npr1-1* (Cao et al., 1994), *ein2-1* (Guzman and Ecker, 1990), *jar1-1* (Staswick et al., 1992), *abi1-1* (Koornneef et al., 1984), *fca-1* (Koornneef et al., 1991), *abi4-1* (Finkelstein et al., 1998), and *Arabidopsis* plants expressing the bacterial *nahG* gene (Bowling et al., 1997). Depending on phenotypes and identities of the particular mutations or insertions, a variety of screening methods employing

hormonal response and PCR-based marker assays were used to isolate double mutants. SALK line N572213 contains a T-DNA insertion inside the cytosolic OAS-TL and was ordered from ABRC (Alonso et al., 2003). The insertion line has the Col-0 background. To isolate plants homozygous for the T-DNA insertion, DNA samples were prepared from young leaf tissues and PCR amplification was done using two sets of oligonucleotide primers. The T-DNA left border primer (PrRuG659 TGGTTCACGTAGTGGGCCATCG) was combined with a gene specific primer PrRuG761 (TACACCAATGGAGTGTTCCCAATCA) to check the presence of the T-DNA insertion inside the targeted gene. PrRuG761 was combined with PrRuG760 (CTATGATCCTTCCGGTGGTGAGAA) to check the presence of the wild type gene. PCR was performed using standard procedures. Plants containing the PrRuG659/761 PCR product but not the PrRuG760/761 PCR product, were homozygous for the T-DNA insertion and were designated as *old3-T* and used in the experiments described.

Unless otherwise indicated, plants were grown in an organic-rich soil (TULIP PROFI No.4, BOGRO B.V., Hardenberg, The Netherlands) under the conditions described by Jing et al. (2002). For cysteine culture and cadmium tolerance assay, seeds were surface-sterilized and kept at 4 °C for 3 days and germinated on solid MS medium containing 0.8% agar with and without cysteine or cadmium chloride at the indicated concentrations.

Map-based cloning and complementation test

Map-based cloning was carried out as described in Chapter 4. DNA was isolated as described above. SNPs were selected, and primers were designed according to Drenkard et al. (2000). In total 5000 recombinants were used for fine mapping. *Agrobacterium*-mediated transformation was performed to further confirm the mutation (Clough and Bent, 1998). The transformants were selected based on basta herbicide resistance.

Oligonucleotide primers PrRuG760 and PrRuG775 (GATGGAAGCAAAGACGCAATGTAACTAA) were used to amplify the cytosolic OAS-TL gene in L*er*-0, *old3-1ODD* and *old3-1OLD3ODD* spanning the nucleotide change caused by *old3-1*. This primer combination gave a PCR product of 2928bp. After Sau96I restriction, the L*er*-0 copy gave a restriction pattern of three bands consisting of 1813, 718 and 319bp, whereas *old3-1ODD* gave 2210 and 718bp bands. The primers PrRuG848 (ACCTCGTGATAATCAATTGTTCCAGCGGAAT) and PrRuG849 (GAATGAACCGTGAGCTGATGGTGCTGGTGTTA) were designed to span the inter-gene region between *AT3G44610* and *AT3G44620* and were used for PCR amplification and identification of the *ODD* polymorphism between different Arabidopsis accessions.

BAC library screen

An *Arabidopsis* L*er*-0 binary BAC library (010-ATL-BI) equivalent to 14.6x the genome was screened. The features of the library were described by Chang et al. (2003). The 10-kb PrRuG848/849 PCR fragment was ^{32}P labelled and hybridised to the filters containing the binary BAC library clones as described (Jing et al., 2002). Twelve clones 5O18, 7N10, 8L11, 9O19, 10F6, 10G16, 20O12, 24H10, 26N21, 27C3, 29B24, and 30G9 showed the strongest hybridisation signals. Two independent clones (10G16 and 24H10) were transformed to *Agrobacteria,* which were subsequently used to transform *old3-1odd* plants via the floral dip method. Seeds were harvested and transformants were selected on agar plates containing 50µg/ml kanamycin.

The survivors were transferred to soil and the transgenic presence of the kanamycin gene was confirmed by PCR amplification. The transformants exhibited *old3-1* phenotypes.

Physiological analyses

Chlorophyll content and ion leakage was measured as described by Jing et al. (2002). For total sulphur, anions and thiols measurement, plants were grown in soil saturated with 25% Hoagland solution for 27 days at 25°C and 70% relative humidity and at a light intensity of 250µmolcm^{-2}s^{-1}. The light cycle was set at a 16-h white light/8-h dark photoperiod. The above ground parts of plants were harvested for fresh weight and dry weight measurement. For each line four replicates of 17 plants were used for quantification. For the total and anion sulphate content data of *old3-1ODD* mutants, one replicate of 50 plants was used for analysis due to the small size of plants. The dry plant material was used for total sulphur measurement using the barium sulphate precipitation method (Jones, 1995) and for anion measurement using HPLC-based methods (Tauze et al., 1996). Parts of the fresh samples were used for total water-soluble non-protein sulfhydryl (SH) compounds following the method described by de Kok and Graham (1989).

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Summary

Senescence, often referred to as ageing in animal biology, is a complex biological phenomenon. This is well illustrated by the isolation and identification of hundreds of senescence-associated genes. In many model organisms across different kingdoms, genes that can substantially alter lifespan have been isolated demonstrating that senescence is subject to genetic regulation. Senescence in plants is exemplified by leaf senescence, a process that occurs at the final phase of leaf development and is marked by massive translocation of nutrients from senescing leaves to other parts of the plants. In this thesis, the isolation and characterisation of genes that are involved in the regulation of leaf senescence is described. The model plant *Arabidopsis* was chosen owing to its obvious advantages of a short life cycle, small size, and the availability of whole genome sequence. The work presented allows for making several important notions.

Multiple genetic loci are involved in the regulation of ethylene-induced senescence
Hormonal modulation is one of the prominent strategies employed by both animals and plants to regulate senescence. Almost all the identified phytohormones are involved in leaf senescence one way or another. Ethylene is a gaseous endogenous plant hormone that plays essential roles in many facets of plant growth, development and stress responses. A complex relationship between leaf senescence and ethylene was observed. The investigation on how the induction of senescence by ethylene changes with leaf age, shows that ethylene does not induce leaf senescence before a certain developmental stage (Chapter 2). These results are consistent with the notion that leaf age determines the effect of ethylene on leaf senescence. However, the study, on how the induction of senescence by ethylene changes with the time of ethylene exposure, found that within a certain range of ethylene exposure times, plants with longer ethylene exposure time exhibit stronger senescence symptoms, but when treated with extra long ethylene exposure, plants display reduced responses to ethylene's induction of senescence. Thus, changing ethylene exposure time can substantially alter leaf senescence in plants with the same final age (Chapter 3). It is shown that there is an active cross-talk between leaf age and ethylene to influence each other's action in senescence. Analyses on ethylene-induced senescence in *old* (*onset of leaf death*) mutants indicated that many genes are involved in regulating the induction of senescence by ethylene (Chapter 3). Thus, the regulation of senescence depends on a complex interaction between leaf age and ethylene involving multiple genetic loci.

The senescence window concept can be employed to explain the developmental features of leaf senescence
Leaf senescence consists of the last phase of the life cycle of a leaf and leaf age is the best predictor of when senescence is initiated. The gene expression profiles of senescing leaves are distinctly different from those of young leaves as envisaged by the time specific up and down regulation of the expression of senescence-associated genes (*SAG*s). Thus, leaf senescence is a developmental programme because of the existence of age-specific gene action. The senescence window concept separates leaf development into distinct phases depending on whether and how senescence can be induced (Chapter 1). This concept is illustrated by the effects of ethylene on senescence (Chapters 2 and 3). During the initial leaf growth and development (the never-senescence phase), senescence is not induced by ethylene

treatment. Thereafter, ethylene can induce senescence in a leaf-age and ethylene-duration dependent manner (the ethylene-modulated senescence phase) (Chapter 2). The transition from the never-senescence phase to the ethylene-modulated senescence phase involves the action of multiple genetic loci (Chapter 3). In this sense, the senescence window concept provides an explanation for the developmental features of leaf senescence. Such developmental features are also evident in animal ageing whose modulation by several genetic pathways appears to fit to a senescence window concept (Chapter 1). Thus, it seems that the senescence window concept has universal implications in explaining the developmental features of senescence.

Senescence regulatory genes have general functions in plant metabolism and development

During senescence, the ordered occurrence of sequential events leads to the death of the leaf cells. Thus, leaf senescence has been considered a typical developmental programmed cell death process. It has long been assumed that specific senescence regulatory genes (genes that only work to regulate senescence and have no other functions) must have evolved to control leaf senescence. However, the results obtained from this study show that *OLD1* and *OLD3* have more functions than only regulating senescence (Chapters 4 and 5). *old1* is allelic to *cpr5* (*constitutive expressor of pathogen resistance 5*)/*hys1* (*hypersenescence 1*) and displays distinct functions throughout plant development. It controls seedling development via independently coordinating multiple hormonal signals and it represses spontaneous lesion formation and early leaf senescence in adult plants. Moreover, it promotes senescence at late development as envisaged by the results obtained with ectopic expression of *OLD1/CPR5*. Thus, *OLD1/CPR5* is involved in many aspects of plant development, and senescence regulation is only part of its multiple functions. Even for the control of leaf senescence, it exhibits two opposite effects depending on the developmental stage. Thus, *OLD1* does not appear to be a specific senescence regulatory gene. Molecular cloning of *old3* showed that it encodes a mutated cytosolic *O*-acetylserine (thiol) lyase (OAS-TL) (Chapter 5). In addition to early leaf senescence, *old3* mutants display an altered sulphur balance and an increased cadmium tolerance. Although the exact molecular mechanisms through which *old3* induces senescence is not clear, *old3* does seem to have dual functions in cysteine synthesis and leaf senescence. Thus, the isolated senescence regulatory genes appear to possess general functions in plant metabolism and development.

This notion is consistent with other studies, which have described several senescence regulatory genes with additional functions. For instance, *ORE9* regulates lateral shoot branching, *SAG101* is involved in fatty acid metabolism, *GIN2/HXK1* controls sugar sensing, seedling growth, and stress response. Genes involved in the regulation of animal ageing appear to have basic function in cellular maintenance and repair as well. So far, it seems that genes that specifically evolved to regulate senescence may not exist. Indeed, organisms are not programmed for senescence or death but are programmed for survival.

Leaf senescence and animal ageing share conserved regulatory strategies but diverge in particular molecular pathways

There is a debate whether leaf senescence in plants can be compared with animal ageing. First, leaf senescence is a cell death event at the organ level, whereas ageing is studied at the whole organism level. Furthermore, leaf senescence is associated with the relocation of useful nutrients and appears to be essential for plant survival. Due to such a strong adaptive

advantage, leaf senescence was thought to be different from animal ageing, which is considered a physiological deterioration process and is non-adaptive. However, both leaf senescence and animal ageing display strong developmental features, which result from the age-specific gene action (Chapters 1). Furthermore, during leaf senescence and animal ageing, *SAG*s have been found and they similarly include genes that are involved in protein and lipid degradation, transport, cellular stress- and defence-related responses, and transcriptional regulation. These similarities suggest that leaf senescence and animal ageing may share common regulatory strategies. Indeed, hormones, metabolic flux, reactive oxygen species and protein degradation are prominent strategies employed by plants to control leaf senescence and by animals to control ageing (Chapter 1). In addition, *OLD1/CPR5* appears to be a typical senescence regulatory gene that exhibits pleiotropic functions throughout development as predicted by Evolutionary Theories of Senescence that were developed from the observations made on the survivorship of wild animals (Chapter 4). Thus, the same classic evolutionary theories seem to be able to serve as guidelines to study the molecular basis of senescence evolution in plants. Nonetheless, they diverge in the particular molecular pathways. One particular example is the modulation of senescence by hormones. In animals, insulin/IGF (insulin growth factor)-1 like signalling is a dominant pathway, whereas plants employ an array of phytohormones. *OLD1/CPR5* shares no similarities with any ageing regulatory genes at the nucleotide and amino acid levels (Chapter 4).

In summary, the mutational analysis employed in this study is an effective approach to isolate senescence regulatory genes in plants. The developed senescence window concept appears to be able to describe age-specific gene action involved in senescence regulation. The presence of *old* mutants with a wide spectrum of altered leaf senescence phenotypes provides a genetic basis for the further dissection of the molecular mechanisms of leaf senescence. Cloning and characterisation of *OLD1/CPR5* and *OLD3* allowed the regulation of leaf senescence to be viewed from fresh perspectives. The Evolutionary Theories of Senescence will certainly benefit the identification of genes for senescence regulation. Future research focusing on cloning more *OLD* genes and dissecting their involvement in senescence, in parallel with genome-wide tools, shall help unravel the complex regulatory network of leaf senescence.

Samenvatting

Senescence, vaak aangeduid met veroudering in biologie van dieren, is een complex biologisch verschijnsel. Dit wordt goed geïllustreerd door het feit dat honderden senescence geassocieerde genen geïsoleerd en geïdentificeerd zijn. Uit vele model organismen, verdeeld over verschillende rijken, zijn genen geïsoleerd die de levensduur aanzienlijk kunnen veranderen en laten daarmee zien dat senescence onderworpen is aan genetische regulatie. Als voorbeeld voor senescence bij planten is gekozen voor bladveroudering, een proces dat plaats vindt gedurende de laatste fase van de bladontwikkeling en wordt gekenmerkt door grootschalige verplaatsing van voedingsstoffen van verouderende bladeren naar andere delen van de plant. In dit proefschrift wordt de isolatie en karakterisering van genen beschreven die een rol spelen bij blad veroudering. De modelplant *Arabidopsis* werd gekozen omdat deze een korte levenscyclus heeft, klein van stuk is en bovendien is de DNA basenvolgorde van het hele genoom bekend. Het hier gepresenteerde onderzoek geeft nieuwe inzichten naar de regulatie van senescence.

Meerdere genetische loci spelen een rol in de regulatie van de ethyleen geïnduceerde senescence

Hormonale besturing is een van de vooraanstaande strategieën die door zowel dieren als planten gebruikt worden om senescence te reguleren. Bijna alle geïdentificeerde fytohormonen spelen op de een of andere manier een rol in blad senescence. Ethyleen is een gasvormig endogeen plantenhormoon dat een essentiële rol speelt in vele facetten van plantengroei, ontwikkeling en reactie op stress. Een complex verband werd waargenomen tussen bladveroudering en ethyleen. Het onderzoek naar hoe de inductie van senescence door ethyleen verandert met de leeftijd van het blad laat zien dat ethyleen geen bladveroudering induceert voor een bepaald ontwikkelingsstadium (Hoofdstuk 2). Deze resultaten zijn in overeenstemming met het idee dat de leeftijd van een blad het effect van ethyleen op bladveroudering bepaalt. Het onderzoek toonde aan dat de inductie van veroudering door ethyleen verandert met de tijdsduur van blootstelling aan ethyleen: planten, welke werden blootgesteld aan langere periodes in ethyleen vertoonden sterkere verouderingssymptomen, echter wanneer behandeld werd met extra lange periodes met ethyleen, vertoonden planten een verminderd effect van ethyleen op de inductie van veroudering. Dus verandering van de periode van blootstelling aan ethyleen kan bladveroudering in planten met dezelfde uiteindelijke leeftijd sterk beïnvloeden (Hoofdstuk 3). Het werd aangetoond dat er een actieve wisselwerking bestaat tussen de leeftijd van een blad en ethyleen om elkaar´s effecten op veroudering te beïnvloeden. Analyses van ethyleen geïnduceerde veroudering in *old* (*onset of leaf death*, *aanvang van blad afsterving*) mutanten laat zien dat vele genen een rol spelen bij de regulatie van de inductie van veroudering door ethyleen (Hoofdstuk 3). Dus de regulatie van veroudering hangt af van een complexe communicatie tussen bladleeftijd en ethyleen waarbij meerdere genetische loci een rol spelen.

Het concept van een senescence-venster kan gebruikt worden om de ontwikkelingskenmerken van bladveroudering te verklaren

Bladveroudering is de laatste fase van de levenscyclus van een blad en de leeftijd van een blad is de beste voorspellende indicator voor het tijdstip waarop veroudering in gang wordt

gezet. De genexpressie profielen van verouderende bladeren zijn duidelijk verschillend van die van jonge bladeren zoals duidelijk blijkt uit de in de tijd gereguleerde expressie van aan veroudering geassocieerde genen (senescence-associated genes, *SAG*'s). Dus bladveroudering is een door de ontwikkeling gestuurd programma omdat er leeftijdsspecifieke genactiviteit bestaat. Het senescence-venster concept scheidt bladontwikkeling in verschillende fasen afhankelijk van of en hoe veroudering kan worden geïnduceerd (Hoofdstuk 1). Dit concept wordt geïllustreerd door de effecten van ethyleen op veroudering (Hoofdstukken 2 en 3). Gedurende het begin van bladgroei en ontwikkeling (de nooit-verouderingsfase), wordt veroudering niet geïnduceerd door ethyleenbehandeling. Daarna kan ethyleen veroudering induceren op een manier die afhankelijk is van de leeftijd van het blad en de tijdsduur van de ethyleen behandeling. Dit is de tweede fase, de door ethyleen te modificeren verouderingsfase (Hoofdstuk 2). De overgang van de nooit-verouderingsfase naar de door ethyleen te modificeren verouderingsfase brengt de activering van verschillende genetische loci met zich mee (Hoofdstuk 3). In deze zin levert het senescence-venster concept een verklaring voor de ontwikkelingskenmerken van bladveroudering. Zulke ontwikkelingskenmerken komen ook duidelijk bij dierlijke veroudering voor, waarbij de regulatie door verschillende genetische richtingsprocessen lijkt te passen in een senescence-venster concept (Hoofdstuk 1). Dus het lijkt dat het senescence-venster concept algemeen toepasbaar is om de ontwikkelingskenmerken van veroudering te verklaren.

Verouderingsregulatie genen hebben algemene functies in plantmetabolisme en ontwikkeling

Tijdens het verouderingsproces leidt het geordend voorkomen van opeenvolgende gebeurtenissen tot het afsterven van bladcellen. Daarom wordt bladveroudering beschouwd als een typisch door de ontwikkeling geprogrammeerde vorm van celdood. Men heeft sinds lange tijd aangenomen dat specifieke verouderingsregulatie genen (genen die alleen werkzaam zijn om veroudering te reguleren en geen andere functie hebben) moeten zijn ontstaan om bladveroudering te sturen. De resultaten van dit onderzoek laten echter zien dat *OLD1* en *OLD3* meer functies hebben dan alleen maar de regulatie van veroudering (Hoofdstukken 4 en 5). *old1* is een allel van *cpr5* (*constitutive expressor of pathogen resistence 5,* ofwel *hys1, hypersenescence 1*) en vertoont verschillende functies tijdens de gehele ontwikkeling van de plant (Hoofdstuk 4). Het bestuurt de ontwikkeling van de zaailing via onafhankelijk gecoördineerde meervoudige hormoon signalen en het onderdrukt de vorming van spontane laesies en vroege bladveroudering in volwassen planten. Bovendien bevordert het veroudering gedurende de late ontwikkeling zoals aangetoond werd door de resultaten verkregen met de ectopische expressie van *OLD1/CPR5*. *OLD1/CPR5* speelt dus een rol in vele aspecten van plant ontwikkeling en de regulatie van veroudering is slechts een deel van zijn meervoudige functies. Zelfs voor de controle van bladveroudering vertoont het twee tegenstrijdige functies die afhangen van het ontwikkelingsstadium. *OLD1* blijkt zodoende geen specifiek verouderingsregulatie gen te zijn. Moleculaire klonering van *old3* liet zien dat het codeert voor een gemuteerde cytosolische O-acetylserine (thiol) lyase (OAS-TL) (Hoofdstuk 5). Naast vroege bladveroudering vertonen *old3* mutanten een veranderde zwavelbalans en een verhoogde tolerantie voor cadmium. Hoewel het exacte moleculaire mechanisme waarmee *old3* veroudering induceert, niet duidelijk is, lijkt *old3* een dubbele functie te hebben in cysteinesynthese en bladveroudering. De geïsoleerde verouderingsregulatie genen bezitten algemene functies in plantmetabolisme en ontwikkeling.

Dit begrip is in overeenstemming met andere onderzoekingen die verschillende verouderingsregulatie genen hebben beschreven met bijkomende functies. Bijvoorbeeld, *ORE9* reguleert laterale vertakking van de stengel, *SAG101* speelt een rol in het vetzuurmetabolisme, *GIN2/HXK1* controleert waarneming van de aanwezigheid van suiker, groei van de zaailing en reactie op stress. Genen die een rol spelen bij de regulatie van het verouderingsproces bij dieren blijken tevens een basis functie te hebben in onderhoud en reparatie van de cel. Tot nu toe, schijnt het dat genen die een specifieke rol hebben in de regulatie van veroudering, eigenlijk niet bestaan. Trouwens, organismen zijn niet geprogrammeerd om te verouderen of dood te gaan maar zijn geprogrammeerd om te overleven.

Bladveroudering en veroudering bij dieren hebben geconserveerde regulatie mechanismen gemeenschappelijk, maar verschillen in speciale moleculaire richting gevende processen. Er bestaat een meningsverschil over het feit of bladveroudering in planten vergeleken kan worden met veroudering in dieren. In de eerste plaats is bladveroudering een gebeurtenis van celdood op orgaan niveau, terwijl veroudering bij dieren wordt bestudeerd op het niveau van het hele organisme. Verder is bladveroudering verbonden met de verplaatsing van nuttige voedingsstoffen en schijnt essentieel te zijn voor de overleving van de plant. Door dit sterke adaptieve voordeel, werd bladveroudering beschouwd anders te zijn dan veroudering bij dieren. Veroudering bij dieren wordt beschouwd als een fysiologisch desintegratie proces en is niet adaptief. Echter zowel bladveroudering als veroudering bij dieren vertonen duidelijk ontwikkelingsbiologische kenmerken die het resultaat zijn van leeftijdspecifieke gen activiteit (Hoofdstuk 1). Bovendien werden tijdens bladveroudering en veroudering bij dieren, *SAG´*s gevonden en die omvatten eveneens genen die een rol spelen in eiwit en lipide degradatie, transport, aan cellulaire stress en afweer verwante reacties en transcriptie regulatie. Deze overeenkomsten suggereren dat bladveroudering en veroudering bij dieren algemene regulatie strategieën gemeenschappelijk kunnen hebben. Inderdaad, hormonen, metabolische flux, moleculen met reactief zuurstof en eiwitdegradatie zijn toonaangevende strategieën die door planten gebruikt worden om bladveroudering te controleren en door dieren om algemene verouderingsprocessen te sturen (Hoofdstuk 1). Bovendien blijkt *OLD1/CPR5* een typisch verouderings-regulatie gen te zijn die gedurende de hele ontwikkeling pleiotropische functies vertoont zoals voorspeld door de evolutionaire theorieën over veroudering die ontwikkeld zijn door waarnemingen gedaan aan de overleving van dieren in het wild (Hoofdstuk 4). Dus dezelfde klassieke evolutionaire theorieën schijnen te kunnen dienen als richtlijnen om the moleculaire basis van verouderingsevolutie in planten te bestuderen. Echter, zij verschillen in de specifieke moleculaire richtingsprocessen. Een speciaal voorbeeld is de besturing van veroudering door hormonen. In dieren is de op de insuline/IGF (insuline groei-factor) lijkende signalering een overheersend richtinggevend proces, terwijl planten een scala aan fytohormonen gebruiken. *OLD1/CPR5* vertoont op nucleotide en aminozuur niveau geen gelijkenis met enig verouderingsregulerend gen bij dieren (Hoofdstuk 4).

Samengevat, de mutatie analyse gebruikt in dit onderzoek is een effectieve benadering om verouderingsregulerende genen in planten te kunnen isoleren. Het ontwikkelde senescence-venster concept lijkt in staat te zijn leeftijdsspecifieke gen activiteit te beschrijven die een rol speelt in de regulatie van veroudering. De aanwezigheid van de *old* mutanten met een wijd spectrum aan fenotypen met een veranderd bladverouderingspatroon levert een genetische basis voor het uit elkaar halen van het moleculaire mechanisme van bladveroudering. Klonering

en karakterisering van *OLD1/CPR5* en *OLD3* stelt ons in staat de regulatie van bladveroudering vanuit een vernieuwd perspectief te bezien. De evolutionaire theorieën van veroudering zullen zeker nuttig zijn voor de identificatie van genen die veroudering reguleren. Toekomstig onderzoek gericht op het kloneren van meer *OLD*-genen en het ontrafelen van hun rol in veroudering, samen met het ontwikkelen van instrumenten voor genoomanalyse, zal helpen om het complexe regulatie netwerk van bladveroudering verder te ontrafelen.