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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.**

Key words: **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.

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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 in 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; Nam, 1997; Gan and Amasino, 1997; Buchanan-Wollaston, 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 provides 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 in a large array of genetic loci. This complication has 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 research outside the plant field shows that single gene mutations can substantially alter the lifespan of 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 advances in identifying senescence regulatory genes in recent years, thanks to molecular genetic studies performed with *Arabidopsis* and the completion of its genome sequencing project. Emerging evidence now allows us to glimpse 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 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 field of leaf senescence.

Ageing Strategies in Yeast and Animals

Like many other biological processes, our understanding of ageing has been greatly advanced through studies of short-lived laboratory models. Many mutations that change lifespan have been isolated from yeast and animals, and comparative studies have shown 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 system represents the conserved hormone regulation of lifespan (Kenyon, 2001; Finch and Ruvkun, 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, to adjust growth and development, and to control 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 form of 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 (Merry, 2002; Lin et al., 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 the 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 an imbalance between deterioration resulting from reactive oxygen species (ROS) and protection by antioxidants, and that 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). Homologous and non-homologous recombination pathways are involved in the repair of DNA double strand break and mutations in genes in both pathways have 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 section, we present molecular genetic evidence to show that regulation of ageing in plants shares similarities 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. Until now, all the identified phytohormones are involved in leaf senescence in one way or another. Among the five classic hormones, the roles of ethylene and cytokinin have long been established. Besides, jasmonic acid, salicylic acid, nitric oxide, and brassinosteriod are also implicated in regulating leaf senescence.

The role of ethylene in leaf senescence has been 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 senescence (Bleecker et al., 1988; Oh et al., 1997; Chao 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 also play a master regulatory role in leaf senescence. While increasing cytokinin production could delay 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 cyto-

kinin signalling (Hwang et al., 2002; Hutchison and Kieber, 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 cytokinin involvement.

Jasmonates (JAs) have been 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 photosynthesis-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 under the same conditions. Taken together, these studies indicate that jasmonates have a role in promoting leaf senescence. However, in none of the isolated mutants that are impaired in JA biosynthesis or signalling (Berger, 2002) were aberrant phenotypes in leaf senescence reported, suggesting that jasmonates are not essential. In addition, transgenic plants that either over-express allene oxide synthase, jasmonic acid carboxyl methyltransferase, or under-express 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 senescence, and the role of jasmonates in leaf senescence is still debatable.

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

Metabolic Flux

The photoautotrophic nature of plants makes them fundamentally different from animals. Their energy input depends on available photosynthetic activity, light and CO_2 and altering 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 resources. In *Rubisco* antisense tobacco plants, less dry weight and chlorophyll content was achieved than in 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 senescence was markedly prolonged resulting in extended leaf longevity. This pattern is similar to one of the stay-green mutants described in pea (Thomas and Howarth, 2000). 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 content, as in the *Rubisco* antisense plants, and more importantly, 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 the 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 was substantially delayed (Ludewig and Sonnewald, 2000). In the senescent leaves, the soluble sugars were higher than in the non-senescent leaves, presumably due to the breakdown 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 over-expressing 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 over-expressing *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 altered pathogen resistance was shown to have sugar hypersensitivity and early leaf senescence (Bowling et al., 1997; Yoshida et al., 2002 a).

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 increase, 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 by 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 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 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 oxidative tolerance and longevity. Alteration in non-enzymatic antioxidants could influence senescence (Smirnoff, 2001). In addition, transgenic plants in which the antioxidant enzymes were manipulated, exhibited altered senescence (Orvar and Ellis, 1997; Willekens et al., 1997). Thus 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 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 these pathways were also shown to change senescence (Mou et al., 2000; Mou et al., 2002; Wellesen et al., 2001). 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 demonstrated a link between 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 is shown by the recent identification of *ORE9* (Woo et al., 2001) and *DLS1* (Yoshida et al., 2002 b). 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 a 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 a 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 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 upregulated 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 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 throughout the progression of leaf senescence. Therefore, plants must have excellent mechanisms that guard 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; Liu et al., 2001; Costa et al., 2001). UVH1 is homologous to the XPF component of the 5′ repair en-

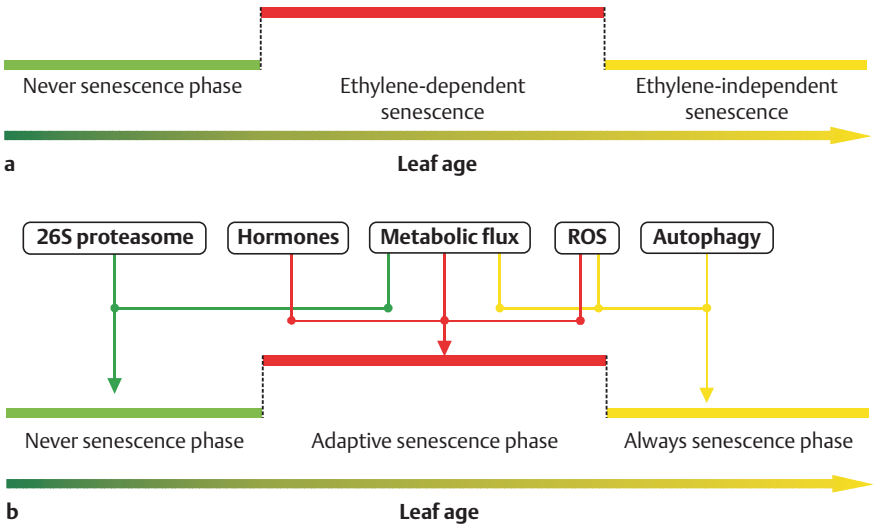

Fig. 1 A schematic representation of the senescence window concept. (**a**) The senescence window as revealed from the effects of ethylene on senescence. (**b**) A tentative model indicating the positions of the major senescence regulators in the senescence window. See text for details.

donuclease, 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, which is required for maintaining leaf longevity. This was the case for a mouse mutant containing the mutated *XPD* that showed premature ageing due to a direct defect in transcription (de Boer et al., 2002). These results demonstrate the link between a 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 also found. One aspect studied was transcriptional regulation by histone deacetylation. In plants, three classes of histone deacetylase 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 be proven. 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 shown to survive 10 generations without telomerase and plants from the last five generations contained severe cellular damage (Riha et al., 2001). Despite these mutations, the late-generation mutants surprisingly had an extended lifespan for both 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 animal 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 humans 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 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 organisms. 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; Hughes et al., 2002; Partridge and Gem, 2002; Partridge, 2001). As discussed below, such age-dependent gene actions can be explained by the senescence window concept.

Fig. **1** shows the senescence window concept which was developed from studies on the interaction between leaf age and ethylene (Grbic and Bleecker, 1995; Jing et al., 2002). Leaf senescence has a distinct tri-phase development in relation to the effect of ethylene (Fig. **1a**). During early leaf growth, ethylene does not induce leaf senescence, and this is termed as 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 will a leaf switch to the second phase, which allows ethylene 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 during the three phases, especially those controlling the transition points of the senescence window, may result in predicted senescence phenotypes. This was experimentally confirmed by isolation and characterisation of ethylene-insensitive mutants and *old* mutants (Grbic and Bleecker, 1995; Jing et al., 2002).

The senescence window concept appears to also apply to other plant hormones. 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 a certain amount 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 also regulates ageing in an age-dependent manner, as shown by a recent elegant study in nematodes (Dillin et al., 2002). When *daf-2* and *daf-16* RNAi treatments were initiated before the 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 lifespan 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 had no effect 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, a drop in 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 between the actions of genes. Apparently, genes working during 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 homeostasis and those that are essential for survival. The genes working in the second phase are presumably those that govern the duration and speed of senescence. The action of these genes allows some plasticity in the progression of senescence, making the second phase more prone to modulation for application purposes. Such genes may be involved in hormonal biosynthesis and signalling. Genes working during the last part of the senescence window may be mainly activated by the second class genes that amplify the effects and start to take action when the second class genes no longer contribute to senescence. 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 (Fig. **1 b**). The ubiquitin proteolysis pathway is placed in the first phase of the senescence window, based on the following evidence: *ore9* and *dls1* mutants showed delayed onset of both natural and hormone-induced leaf senescence, and the proteins targeted for degradation by the 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; Meinhard and Grill, 2001; Delledonne et al., 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 during 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 positioned based on the developmental phases on which they act, which to a certain extent, is similar to proposals to explain ageing in *C. elegans* (Gems, 2000). Although preliminary, the senescence window concept seems to be universal and could 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 survival strategies that have evolved in plants and animals, one being an autotroph, the other a heterotroph. Nevertheless, the special features of plant life forms lead to 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 calorie restriction interventions, free radical theory of ageing and hormonal modulation being the most conspicuous. In this context, there is little difference 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 does senescence involve 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 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 this theory: (1) Genes do not evolve solely to 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 and these 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, which 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 effort is 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 *a priori* 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 parallel with genome-wide approaches, will help to build a complete picture of the regulation of senescence in plants.

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