Chapter 1

Developmental and hormonal control of leaf senescence

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Introduction

What controls the length of life is one of the fundamental biological questions that has been puzzling scientists for centuries. Plants have many life-forms and differ greatly in the maximal life spans (Thomas, 2003). Annual and biennial plants finish life cycles in a single season or in 2 years time, respectively. An age of 4600 years has been recorded for the perennial tree bristlecone pine (*Pinus longaeva*), while some clonal plants can live over 10 000 years (Nooden, 1988). Thus, longevity is a genetically controlled life-history trait.

The phenomenon of leaf senescence can be appreciated by the color changes among deciduous trees and in the ripening of cereal crops in late summer and autumn, which can occur at a global scale to transform the appearance of the earth from space. During leaf senescence, the sum of morphological, physiological and molecular changes is generally referred to as the senescence syndrome, which includes the visible color changes, dismantling of chloroplasts, degradation of RNA, proteins and DNA and translocation of macro/micromolecules from senescing leaves to other parts of the plant, resulting in the death of the leaf (Bleecker and Patterson, 1997).

We propose to examine the regulation of leaf senescence from a genome optimization perspective. We critically analyze the proposed developmental cues that are implicated in initiating leaf senescence. The prominent roles that hormones play during developmental ageing and the initiation and progression of senescence will be reviewed from a molecular point of view based partially on transcriptome data. We discuss the identified potential physiological, biochemical and molecular events during developmental senescence.

Developmental senescence: a plant genome is optimized for early survival and reproduction

In general, a genome has evolved to contain three classes of hereditary information: (1) the basic metabolism and life maintenance program such as photosynthesis, respiration and DNA replication and damage repair; (2) the defense program that regulates plant responses to abiotic and biotic stresses; and (3) the growth and development program that produces an adult plant optimized for reproduction.
(Gems, 2000; de Magalhaes and Church, 2005). From an evolutionary point of view, a genome is selected by the force of natural selection if it facilitates the continuous reproduction. Thus, natural selection optimizes a genome for reproduction and the aforementioned three classes of genome programs will be operating only to ensure normal plant growth and development until reproduction. After reproduction, the force of natural selection declines with age and this leads to the loss of viability and fitness of the whole plant and/or plant organs. This phenomenon is known as disposal of soma as stated in the evolutionary theory of ageing, which is developed from animal ageing studies (Rose, 1991; Kirkwood, 2005). This argues that in the genome there are no specific genetic programs for life span and that longevity is an indirect consequence of genome optimization for reproduction.

However, the annual model plant *Arabidopsis thaliana* has evolved a reproduction program that runs in parallel with the death of the whole plant. Seeds are being produced while the plant starts to senesce, and in this way the plant effectively reutilizes nutrients stored in leaves for the production of seeds. Here, the evolutionary theory of ageing can explain leaf senescence if the disposal of a leaf is considered as an indirect selection for nutrient salvage (Bleecker, 1998). Indeed, selective cell death is well documented during plant development and defense responses. For instance, xyleogenesis, early embryogenesis, pollen tube growth and the hypersensitive response are typical examples. Thus, a common feature of the plant body plan and architecture is that almost all the structural units are disposable for the sake of survival and reproduction. Clearly, the recruitment of nutrients from leaf tissues, which is a prominent feature of the senescence syndrome and results in the death of the leaf, is part of the genome optimization program. Following this line of arguments, we consider that leaf senescence, albeit genetically controlled, is a consequence of natural selection for genome reproduction. Although there are debates concerning whether ageing occurs in plants, or whether whole-plant senescence shares similarities with animal ageing (Thomas, 2002), it has been proposed that developmental ageing resembles animal ageing, especially when leaves on a plant are scaled up and viewed as equivalent of animal individuals. Leaf senescence is a typical postmitotic senescence in plants and its onset shares many similar regulatory strategies with ageing in animals (Gan, 2003; Jing et al., 2003).

We consider it important to view leaf senescence from such an angle. This view helps to explain and model the molecular genetic mechanisms of leaf senescence.
As predicted by the evolutionary theory of ageing, genes with early-life beneficial but late-life deleterious effects and late-acting mutations with purely deleterious effects are important for senescence regulation (Kirkwood and Austad, 2000). In the following part of the chapter, we will show that programs important for life maintenance, stress responses and development are important for the onset and regulation of leaf senescence. We further summarize the recent progress in examining the interactions between leaf development and ethylene as an example to present the approaches we think are necessary to understand the complex process of developmental senescence.

**Developmental processes that regulate leaf senescence**

When a plant is grown in an environment with sufficient nutrition, away from pathogen attacks and free of abiotic stresses such as darkness, drought, extreme temperature, UV-B and ozone, leaf senescence is ultimately initiated and progresses in a leaf age dependent manner (Gan and Amasino, 1997; Quirino et al., 2000). For monocarpic plants the regulation of senescence is under correlative control and the onset of whole-plant senescence is initiated by the developing reproductive sink, which remobilizes nutrients from the vegetative tissues (Nooden, 1988). In soybean and wheat the removal of reproductive structures usually delays leaf and whole-plant senescence (Nooden, 1984; Srivalli and Khanna-Chopra, 2004). Thus genomes of monocarpic plants are optimized for reproduction, which determines the onset of leaf and whole-plant senescence. Although whole-plant senescence in Arabidopsis is controlled by the reproductive structures as well (Nooden and Penney, 2001), only a weak correlation exists between the appearance of reproductive structures and the onset of leaf senescence. *Arabidopsis* leaves have a defined life span and senesce even under ideal growth conditions, which is due to the developmental programs underlined by the genome (Hensel et al., 1993; Jing et al., 2002). Thus, here the onset of leaf senescence is governed mainly by age-related changes. Strategies employed in animals and humans seem to have been equally used in plants, such as hormonal modulation as discussed in the next section, reactive oxygen species (ROS), metabolic flux especially sugar and nitrogen signaling and protein degradation. Readers are advised to refer to several recent reviews for detailed
discussion on them (Gan, 2003; Jing et al., 2003; Lim et al., 2003; Lim and Nam, 2005;). In this section, we will only briefly elaborate on those strategies.

**Reactive oxygen species**

Leaf senescence and the expression of various senescence-associated genes (SAGs) were promoted in old leaves upon exposure to UV-B, ozone or treatment with catalase inhibitors (Miller et al., 1999; John et al., 2001; Navabpour et al., 2003). In contrast to animal ageing and plant hypersensitive responses during plant–pathogen interactions in which mitochondria are the generator of ROS (Finkel and Hollbrook, 2000; Lam et al., 2001; Biesalski, 2002), the main ROS source in a senescing leaf is chloroplasts (Quirino et al., 2000). This is consistent with the observation that knockout of a chloroplast genome encoded ndhF gene, one of the components of Ndh complex involved in chlororespiratory electron transport chain, delayed leaf senescence in tobacco (Zapata et al., 2005). ROS can also be generated via lipid oxidation involving membrane-associated NAD(P)H oxidases (Mittler, 2002). This is in agreement with the observed altered senescence phenotypes of Arabidopsis antisense-suppressed phospholipase Dα and SAG101 plants (Fan et al., 1997; He and Gan, 2002) and in plants with defects in fatty acid biosynthesis pathways (Mou et al., 2000, 2002; Wellesen et al., 2001). Thus, ROS generated from various sources are involved in leaf senescence. Several delayed senescence mutants exhibited enhanced tolerance to oxidative stress, indicating that the extended longevity at least in part is due to the attenuated tolerance to ROS (Woo et al., 2004). Thus, the damage generated by ROS can be an important age-related change that will eventually result in the onset of leaf senescence.

**Metabolic flux**

One of the distinct features during leaf senescence is the clear metabolic shift from primary catabolism to anabolism (Smart, 1994; Buchanan-Wollaston, 1997). The number of catabolic genes highly expressed in senescing leaves is almost twofold of that of anabolic genes (Guo et al., 2004). Carbon and nitrogen supplies are the two key components that reflect the control of metabolic flux on leaf senescence. An elevated CO₂ level hastened the drop in the photosynthetic activities and induced leaf senescence (Miller et al., 1997; Ludewig and Sonnewald, 2000), whereas in Rubisco antisense tobacco plants and Arabidopsis ore4-1 mutants, less dry weight and
chlorophyll content were achieved than in the wild type at maturity, resulting in a prolonged leaf longevity (Miller et al., 2000; Woo et al., 2002). Thus, carbon supply achieved through photosynthesis is important for the onset of leaf senescence (Hensel et al., 1993). Carbon supply may directly alter the sugar sensing and signaling, which has been shown to regulate leaf senescence as envisaged in the gin2 mutant that has a lesion in a hexokinase gene (Moore et al., 2003). The cpr5/hys1 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., 2002b). Furthermore, glucose (carbon supply) was shown to induce early leaf senescence when combined with low, but not high nitrogen supply (Wingler et al., 2004), indicating the importance of carbon–nitrogen balance. Nitrogen starvation can induce premature leaf senescence, perhaps mainly through modulating the autophagy functions (see below).

Protein degradation
As one of the essential activities in plant life, protein turnover involves selective and bulk removal of proteins in many processes, such as the degradation of specific regulatory gene products, the maintenance of free amino acids, the elimination of malfunctioning proteins and nutrient recycling (Smalle and Vierstra, 2004; Thompson and Vierstra, 2005). The identification of Arabidopsis ORE9 as an F-box protein (Woo et al., 2001) and DLS1 as an arginyl-tRNA:protein arginyltransferase (ATATE1), which is involved in the N-end rule pathway (Yoshida et al., 2002a), demonstrated the importance of the selective protein removal route mediated by the ubiquitin-mediated proteolysis pathway via 26S proteasome in leaf senescence. Mutations in these two genes resulted in delayed senescence, suggesting that the degraded products targeted by ORE9 and DLS1 are positive regulators of leaf senescence, or that the nondegraded products delay senescence. The bulk protein turnover is mainly achieved through vacuolar autophagy. The analyses of two autophagic mutants apg7 and apg9-1 demonstrated the importance of autophagy in senescence regulation (Doelling et al., 2002; Hanaoka et al., 2002). Many more components involved in autophagy formation, conjugation and targeting to vacuoles have been studied through mutational analyses in Arabidopsis (Surpin et al., 2003; Yoshimoto et al., 2004; Thompson et al., 2005; Xiong et al., 2005). In general, knockout of these components affected the survival under carbon and nitrogen starvation conditions.
and hastened leaf senescence under normal growth conditions. Interestingly, the mRNA and protein levels of autophagy genes are senescence enhanced, suggesting that autophagy is an important aspect of the senescence syndrome. A major group of SAGs encode cysteine proteases (Bhalerao et al., 2003; Guo et al., 2004). For instance, RD21 remains in the vacuole as inactive aggregate and becomes active during senescence by the cleavage of its C-terminal granulin domain (Yamada et al., 2001). Recently, a novel type of senescence-associated vacuole (SAV) has been observed in Arabidopsis and soybean which contains many proteolytic enzymes such as SAG12 (Otegui et al., 2005). The development of SAVs appears to be differentially regulated from vacuole autophagy that is actively involved in leaf senescence (Doelling et al., 2002; Hanaoka et al., 2002). Thus, different vacuoles are functioning during senescence and play a prominent role in macromolecule degradation (Matile, 1997). Furthermore, a chloroplast nucleotide encoded protein CND41 was shown to be responsible for the degradation of Rubisco proteins in senescent tobacco leaves (Kato et al., 2004), indicative of the involvement of chloroplast genome in leaf senescence. The macromolecule degradation and nutrient recycling are prominent events during senescence. Thus, it is not surprising that protein degradation, selective or bulk, is important for senescence regulation.

**Hormonal control of leaf senescence**

The senescence program is the final developmental phase of a leaf, which is influenced by several phytohormones, with cytokinin and ethylene having the most extensively documented roles in delaying or inducing leaf senescence, respectively. In addition, other hormones, such as abscisic acid (ABA), auxin, gibberellic acid (GA), jasmonic acid (JA) and salicylic acid (SA), also have effect on the senescence process. In plants, two types of senescence are evident: mitotic senescence and postmitotic senescence (Gan, 2003). Cells in leaves divide only during early development, and thus leaf senescence can be considered postmitotic. Research on the effect of plant hormones on senescence has been started already in the late fifties. The effect of various hormones has been reported for dozens of plant species. The regulation of senescence by cytokinin and ethylene is conserved; however, the action of other hormones varies between plant species. Hormonal signaling pathways show significant overlap, which makes the study of the effect of
single hormones complex. The generally used linear representation of hormonal signaling pathways controlling specific aspects of plant growth and development is too simple. In fact, hormones interact with each other and with a whole range of developmental, environmental and metabolic signals (Beaudoin et al., 2000).

There are three major ways of controlling the responses to hormones in plants: regulation by hormone biosynthesis, through hormone perception and signaling pathways and downstream events leading to selective protein turnover and changes in gene expression. Although Arabidopsis is the model species for plant research for the last 15 years, almost all the physiological information about hormonal control of leaf senescence has been generated in other species. Evidence from hormone mutants in Arabidopsis strongly supports the role of several hormones in leaf senescence. Lately, exciting advances through transcriptome studies have revealed expression data for hormone biosynthesis, signaling and response genes during senescence, and a closer examination revealed a few interesting points. Combination of the physiological and genetic information will help creating a model for hormonal and developmental control of leaf senescence.

Here we try to highlight important findings of several studies that we used to present a model of hormonal regulation of leaf senescence and address remaining questions and leads for future research.

Hormones that delay leaf senescence

*Gibberellic acid*

Gibberellins are diterpenes that promote stem and leaf growth. In some species, GAs also induce seed germination and modulate flowering time and the development of flowers, fruits and seeds (Sun and Gubler, 2004). A biochemical relation between leaf senescence and GA was first reported by Fletcher and Osborne (1965) showing that GA retarded senescence of excised leaf tissue from *Taraxacum officinale* by maintaining chlorophyll levels and RNA synthesis. Another study in Rumex by Goldthwaite and Laetsch (1968) showed that GA could inhibit senescence in leaf disks for several days. Both protein degradation and chlorophyll degradation were delayed 4 days. Even when chlorophyll and protein loss is halfway complete, addition of GA blocks further degradation. A study performed on the leaves of romaine lettuce showed a clear age-related decline in GA levels and absence in senesced leaves.
This decline in GA was caused by the conversion of free GA to a bound inactive form, probably GA glucoside (Aharoni and Richmond, 1978). Moreover, retardation of senescence by kinetin also caused a relatively high level of free GA and absence of bound GA. Mutations in genes controlling GA biosynthesis or perception have no effect on senescence. However, mutations in the F-box protein SLEEPY1 (SLY1), which result in a block of GA-responsive genes (Dill et al., 2004), delay senescence when crossed to abi1 (Richards et al., 2001). Although not extensively described, several reports point to a retarding effect of GA on leaf senescence.

**Auxin**

Auxins are a group of molecules that got their name from the Greek word auxein, which means ‘to grow’. The diversity of the auxin responses is reflected by the existence of multiple independent auxin perception mechanisms in a plant (Leyser, 2002). For soybean it has been shown that the senescence can be retarded by application of auxin (Nooden et al., 1979). During abscission, auxin has been postulated to play a role in reducing the sensitivity of the cells to ethylene (Sexton and Roberts, 1982).

The endogenous auxin levels within Coleus leaves showed a decline with increasing age (dela Fuente and Leopold, 1968). However, a relation between endogenous auxin levels and senescence does not always seem to follow a same pattern (Nooden, 1988). The change in auxin response during ageing is the result not only of decreasing auxin levels, but also of a lower responsiveness to auxin with age (Chatterjee and Leopold, 1965). Since auxin in general seen as a senescence-retarding compound it was a surprise that increased indoleacetic acid (IAA) levels could be detected in S3 phase leaves (Quirino et al., 1999). Since leaves do not senesce uniformly, the authors suggested that auxin levels are selectively increased only in a certain population of cells corresponding to a particular senescence stage. These findings actually correlated with earlier studies that show IAA can induce the production of ethylene which opposes the senescence-retarding effect of IAA in tobacco leaf discs (Aharoni et al., 1979). Interestingly, auxin effectively decreased SAG12 expression, a marker for developmental senescence in a very short period of treatment in detached senescing leaves (Noh and Amasino, 1999). Research performed on glucose signaling revealed that the HXK1 glucose signaling
pathway interacts intimately with the auxin and cytokinin pathways. Glucose concentration and photorespiration rates are important determinants for the onset of senescence. Both cytokinin and auxin are part of a regulatory complex for nutritional status of the plant through HXK1 signaling pathway (Moore et al., 2003). Thus, the role of auxin in the regulation of leaf senescence might be linked with other hormones and metabolic flux.

**Cytokinins**

Cytokinins have the strongest effect of all hormones on the retardation of leaf senescence. It was reported by Richmond and Lang (1957) that application of cytokinin could retard leaf senescence by preventing the chlorophyll breakdown. 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). The drop in cytokinin levels before the onset of senescence is believed to be a key signal for the initiation (Nooden et al., 1990; Gan and Amasino, 1995). Recently, exciting advances have been achieved in dissecting the components involved in cytokinin signaling (Hutchison and Kieber, 2002; Hwang et al., 2002). Among the genes characterized, the receptor CKI1 (cytokinin independent 1) and the Arabidopsis response regulator (ARR) 2 appear to be involved in regulating leaf senescence (Hwang and Sheen, 2001). A more recent study identified an extracellular invertase whose activity is induced during cytokinin-mediated delay of senescence (Balibrea Lara et al., 2004). In transgenic tobacco plants having a $SAG12–IPT$ or $SAG12–KN1$ construct, cytokinin biosynthesis was initiated when SAG12 was induced resulting in a block of the senescence syndrome and delayed leaf senescence significantly (Gan and Amasino, 1995; Ori et al., 1999). When extracellular invertase activity is inhibited, cytokinin no longer can inhibit leaf senescence in transgenic $SAG12–IPT$ lines (Balibrea Lara et al., 2004).

Cytokinin signaling genes such as the type-A ARRs and biosynthesis genes show reduced transcription during leaf senescence (Buchanan-Wollaston et al., 2005). Several microarray studies have been performed to reveal cytokinin-dependent gene expression (Hoth et al., 2003; Rashotte et al., 2003; Kiba et al., 2005). Hoth et al. used an inducible system to assess the effects of endogenous cytokinin levels. The study identified 823 up- and 917 downregulated genes after 24 h of isopentenyltransferase (IPT) induction. Although for these studies the seedling stage
was used, this IPT system offers an attractive system to study the molecular genetics of how cytokinin can delay and/or reverse the senescence process. The study by Rashotte et al. (2003) showed that cytokinin-upregulated type-A ARRs, which were downregulated in senescing leaves (Buchanan-Wollaston et al., 2005), are the primary response genes for cytokinins. Also a cytokinin oxidase (that degrades cytokinins) and several transcription factors were upregulated. Furthermore, cytokinins induce genes encoding ribosomal proteins (Crowell et al., 1990) and photosynthetic genes (Mok and Mok, 2001). Application of cytokinins downregulated several peroxidases, kinases and E3 ubiquitin ligases. The regulation by cytokinin is related to auxin, light and sugar, since application of cytokinin influences the expression of genes involved in these signaling pathways.

In general it can be said that cytokinin stimulates the photosynthetic phase of a leaf. How cytokinin can maintain this phase and delay leaf senescence is still unclear. Nevertheless, the leaves of SAG12–IPT transgenic plants will undergo senescence, thus cytokinin action is limited to a certain developmental phase.

**Hormones that induce leaf senescence**

**ABA**

ABA plays a major role during processes related to seed development and germination, for instance the induction of seed dormancy, the synthesis of seed storage proteins and lipids, the acquisition of desiccation tolerance and the inhibition of the transition from embryonic to vegetative growth (Nambara and Marion-Poll, 2005). In vegetative tissue, ABA plays a role in response to drought to prevent water loss by stomatal closure and maintenance of vegetative growth by inhibiting the transition to reproductive growth. Under nonstressful conditions, ABA in plant cells is maintained at low levels, since ABA inhibits plant growth. In vegetative tissues, ABA levels increase during drought, salt and cold stress (Xiong and Zhu, 2003). Changes in gene expression during water-deficit stress are partially induced by ABA and may promote the ability of a plant to respond and survive or adapt to the stress (Bray, 2002). For long it was thought that ABA inhibits plant growth rather than maintaining plant growth. But in tomato, maize and Arabidopsis it has been shown that ABA maintains shoot growth by inhibiting ethylene production (Sharp, 2002). Moreover,
this interaction might also play a role in early leaf senescence and leaf, flower and fruit abscission (Morgan and Drew, 1997).

Young leaves have the highest ABA levels although this is mainly produced and transported from the older leaves (Zeevaart and Creelman, 1988). During vegetative growth the ABA levels are in general very low; however, in parallel with a decline in free cytokinin and GA just before chlorophyll breakdown in lettuce leaves, an increase in ABA levels has been observed (Aharoni and Richmond, 1978). As soon as the chlorophyll breakdown is initiated, a second, more dramatic increase in endogenous ABA levels is observed. The authors suggest that lowering of GA and cytokinin levels mark the onset of leaf senescence, which results in increased ABA levels when the process has been started. Application of ethylene to the lettuce leaves resulted in a quick drop of GA in 1 day after treatment, but the ABA levels did not show any difference. This might indicate that ABA and ethylene both control different aspects of the senescence syndrome which are mediated through different but partially overlapping signaling pathways. The application of ABA to detached leaves results in a rapid senescence response; however, application to attached leaves has a less pronounced effect. Under low nitrogen conditions and high sugar the abi5 mutant shows delayed senescence. This is consistent with a role for sugar signaling during leaf senescence. ABI5 can be induced by glucose during later stages of development. Expression analysis of ABI5 shows an increase during senescence (Buchanan-Wollaston et al., 2005). The ABA signaling mutants abi2-1 and abi1-1 show signs of early leaf senescence when grown on low nitrogen with glucose and their transcripts increase during senescence (Pourtau et al., 2004; Buchanan-Wollaston et al., 2005). Furthermore, the enzymes controlling ABA synthesis are upregulated during senescence. This indicates that the ABA signaling and biosynthesis pathway is active during leaf senescence. Interestingly the abi4 and abi5 signaling mutants and the aba1, aba2 and aba3 ABA-deficient mutants all are glucose insensitive (Arenas-Huertero et al., 2000). It was noted before that sugar represses photosynthesis-associated genes, which leads to a decline in photosynthesis and eventually in leaf senescence (Bleecker and Patterson, 1997). Thus the onset of leaf senescence by ABA appears to be coupled to metabolic flux changes in Arabidopsis.

Brassinosteroids
Brassinosteroids (BRs), polyhydroxylated steroid hormones, regulate the growth and differentiation of plants throughout their life cycle. In recent years great advances have been made in the understanding of BR signaling (Vert et al., 2005). External application of BR results in premature leaf senescence for several species, but it has not been reported for *Arabidopsis*. The induction of senescence by BRs might be mediated through ROS (Clouse and Sasse, 1998). BR signal transduction takes place at the plasma-membrane-localized receptor kinase, BRI1 (Clouse et al., 1996).

In addition to BRI1, three homologues have been characterized. Downstream of the receptor kinases is BIN2, a negative regulator of the BR pathway. Further downstream act BES1 and BZR1 transcription factors of which BES1 promotes the expression of BR-regulated genes and BZR1 represses BR genes; both are repressed by BIN2 by targeting of BES1 and BZR1 for ubiquitination and subsequent proteasome-dependent degradation (Vert et al., 2005). Interestingly, BRs can induce ethylene biosynthesis genes in mung bean (Yi et al., 1999). Whether BRs also induce ethylene biosynthesis during senescence is a question that remains to be answered.

Mutants in BR biosynthesis and BR signaling do support a role for BR in senescence in *Arabidopsis*. The *det2* (de-etiolated2) mutant is defective in an early step of BR biosynthesis. When grown in light the mutant develops two times more rosette leaves than does the wild type. Chory et al. (1991) observed that wild-type plants showed senescence after 30 days, whereas the *det2* mutant did not show any signs of visible senescence after 49 days. One could argue that the mutant has a severe developmental defect that results in a changed senescence syndrome; however, other severely affected developmental mutants such as *ctr1* still show a normal onset of the senescence program (Kieber et al., 1993). BR mutants can also result in early leaf senescence as has been shown by the *bes1* mutant (Yin et al., 2002).

Looking at the *Arabidopsis* transcriptome of leaf senescence, none of the BR signaling components are identified (Guo et al., 2004). This suggests a minor role for BR during leaf senescence. Transcriptome analysis identified seven genes encoding cell-wall-associated proteins that are upregulated after BR treatment (Goda et al., 2002); these genes were not identified in the transcriptome of senescing leaves (Guo et al., 2004). However, one study revealed the induction of SAGs in *Arabidopsis* by BR (He et al., 2001). Out of 125 enhancer-trap lines screened, 4 showed upregulation of the reporter after BR application.
Although BR mutants show an alternative onset of senescence, molecular genetic evidence of a direct role for BR is still minimal. More studies about the role of BR in senescence are necessary.

_Ethylene_

The gaseous plant hormone ethylene plays an important role in plant growth and development. From seed germination to organ senescence and from cell elongation to defense responses, ethylene plays its part. The diverse role that ethylene plays in growth and development suggests that ethylene action involves expression and interaction of many different genes and their products (Zhong and Burns, 2003). Ethylene has long been seen as the key hormone in regulating the onset of leaf senescence (Zacarias and Reid, 1990). The senescence-delaying hormones like auxin and cytokinin both stimulate ethylene production in romaine lettuce leaves (_Lactuca sativa_ L.), which might account for their limited stay-green properties. The author concluded that the effectiveness of exogenously applied hormones in both enhancing and retarding senescence is greatly affected by the endogenous ethylene concentration of the treated plant tissue (Aharoni, 1989). The role of the ethylene pathway in senescence is demonstrated by several studies. Both ethylene insensitive mutants _etr1-1_ and _ein2/ore3_ showed increased leaf longevities (Grbić and Bleecker, 1995; Oh et al., 1997), and antisense suppression of the tomato 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase resulted in delayed leaf senescence (John et al., 1995). In these cases, however, senescence eventually begins and progresses normally. Exogenously applied ethylene induces premature leaf senescence in _Arabidopsis_. However, constitutive application of ethylene does not change the longevity of the leaves. Both _ctr1_ (constitutive triple response) mutants and _Arabidopsis_ plants grown in the continuous presence of exogenous ethylene did not show premature senescence (Kieber et al., 1993; Grbić and Bleecker, 1995). These results suggest a dynamic regulation of the timing of leaf senescence for which the age-dependent effect of ethylene is utilized.

By making use of an ethylene-induced senescence screen, a large collection of _onset of leaf death (old) _mutants has been identified (Jing et al., 2002, 2005). These mutants confirmed that the effect of ethylene is limited to a range of leaf ages, and that the effect of ethylene on leaf senescence increases with increase in leaf age (Grbić and Bleecker, 1995; Jing et al., 2002). Another piece of evidence supporting
this notion comes from a study treating *Arabidopsis* plants of an identical 24-day end age with various lengths of exogenously applied ethylene (Jing et al., 2005). The results showed that increasing ethylene treatment from 3 to 12 days caused an increase in leaf senescence. Surprisingly, a drop in the number of yellow leaves occurred when a 16-day ethylene exposure was applied. Thus, varying ethylene exposure time can induce different degrees of senescence symptoms in the leaves of an identical end age, suggesting that ethylene can actively stimulate or repress age-related changes that control ethylene-induced leaf senescence. This notion is genetically supported by the altered responses of eight *old* mutants to the various ethylene treatments (Jing et al., 2005). Thus, multiple genetic loci are required to regulate the action of ethylene in leaf senescence.

A transcriptome study of senescent leaves by Guo et al. (2004) identified three mitogen-activated protein kinases (MAPKs), three MAPKKs, nine MAPKKKs and one MAPKKKK. In the *Arabidopsis* genome, 20 MAPKs, 10 MAPKKs, 80 MAPKKKs and 10 MAPKKKKs have been identified. The few components identified of the MAPK signal cascades led the authors to the conclusion that the three MAPKs and three MAPKKs may be at the converging/cross talk points of various signal transduction pathways. One of the identified MAPKs is MPK6, which is a component of the MAPK pathway that controls ethylene signaling in plants (Ouaked et al., 2003). MPK6 is upregulated during osmotic stress but also by other abiotic stresses such as low temperature, low humidity, wounding or oxidative stress, as well as by pathogens (Droillard, 2002).

Transcriptional analyses of ethylene mutants and ethylene-treated plants revealed the molecular actions of ethylene. A study by Zhong and Burns (2003) revealed genes that are regulated by ethylene. They compared treated wild type, *etr1* and *ctr1* with untreated wild type. Ethylene treatment of 24-day-old wild-type plants for 24 h changed the expression of 184 genes. Compared to *etr1-1*, 248 genes were changed in expression level. Untreated wild type compared to *etr1-1* revealed the downregulation of nine genes and one upregulated gene in *etr1-1*. The *ctr1* mutant that did not show any signs of early senescence had 109 genes differentially expressed. Further research on these genes might help understanding the molecular regulation of ethylene-induced leaf senescence. To further assess the regulation of senescence by ethylene, expression of SAGs in *ein2* was compared with that of wild type (Buchanan-Wollaston et al., 2005). Nine percent of the genes that are
upregulated during senescence are at least twofold reduced in ein2. Seventy-seven genes are more than twofold up- or downregulated. Four genes showed upregulation, a lipid transfer protein, a heavy-metal-binding protein and a transcription factor (HFR1). Downregulated are nine transcription factors, cell-wall-degrading proteins and nuclease. Therefore some senescence-related degradation functions may be dependent on ethylene. The generation of the transcriptome data revealed that indeed ethylene controls a subset of SAGs during senescence; however, the importance of the identified genes for the control of leaf senescence remains elusive.

Endogenous ethylene levels are important for the initiation of senescence. However, the age-dependent senescence induction by ethylene limits its control to a specific age range. The transcriptome studies on SAGs induced by ethylene, together with physiological studies, reveal extensive cross talks between ethylene and the other hormones that might be utilized to fine-tune the progression of senescence in an age-dependent way.

**Jasmonic acid**

Jasmonates include jasmonic acid (JA), methyl jasmonate (MeJA) and related compounds and are found in fragrant oils. This group of plant regulators is connected to plant growth and development such as germination and seedling development, flower development, tuberisation, tendril coiling, leaf senescence and fruit ripening (Wasternack and Hause, 2002). The promotional effect of MeJA on senescence was first shown by application to detached oat leaves (Ueda and Kato, 1980). Exogenously applied JA or MeJA resulted in a decreased expression of photosynthesis related genes like Rubisco. Moreover, a change in the polypeptide composition in senescing tissue was observed, which shared similarity with ABA-induced senescence in detached leaves (Weidhase et al., 1987). In plants two JA biosynthetic pathways have been identified; a chloroplast-localized pathway and a cytoplasm localized pathway (Creelman and Mullet, 1995). Exogenous application of JA typically promotes senescence in attached and detached leaves of Arabidopsis but not in the JA-insensitive mutant coi1. Also the endogenous JA levels in senescing leaves increased fourfold as compared to no senescing leaves. Besides an increased JA level during senescence also the enzymes involved in the JA biosynthesis are differentially regulated during senescence (He et al., 2002). The coi1 mutant, which is
impaired in JA signaling, did not show any altered leaf senescence. Also other JA-related mutants do not show any alterations in the senescence program, which challenges the idea that JA plays a role in senescence. However, a study with senescence enhancer-trap lines in *Arabidopsis* showed that JA can induce GUS (β-glucuronidase) expression in 14 out of the 125 lines tested (He et al., 2001). The authors developed a sensitive large-scale screening method and have screened 1300 *Arabidopsis* enhancer-trap lines, which resulted in the identification of 147 lines in which the reporter gene GUS is expressed in senescing leaves but not in nonsenescing ones. Application of senescence-inducing factors showed that only ethylene induced GUS expression in more lines than JA and that ABA, BR, darkness and dehydration were less effective. Based on this, JA appears to be an important senescence-promoting factor.

The identification and cloning of *coi1* resulted in the identification of an F-box protein which shows the involvement of proteasome-dependent protein degradation in JA signaling (Xie et al., 1998). Interestingly, application of MeJA to *Cucurbita pepo* (zucchini) induces senescence in 7-day-old cotyledons. One of the observed effects was on the concentration of endogenous cytokinin levels, which reduced rapidly after MeJA treatment (Ananieva, 2004). A drop in cytokinin levels is necessary before senescence can be initiated; whether MeJA can directly or indirectly antagonize cytokinin levels remains to be answered.

Transcriptome analyses of MeJA-treated seedlings showed a self-activation of JA biosynthesis and cross talk with other hormones (Sasaki et al., 2001). Although the *coi1* mutant does not show any visual senescence defects, a transcriptional analysis showed that 12% of the identified developmental senescence genes are not expressed during senescence of *coi1* (Buchanan-Wollaston et al., 2005). In addition, certain genes that are downregulated in the *coi1* mutant also appear to be downregulated in the *ein2* or *nahG* mutants. This further demonstrates the importance of the JA pathway during leaf senescence.

**Salicylic acid**

SA, a phenolic compound, has been identified as a key-signaling molecule in various plant responses to stress, like pathogen invasion (Glazebrook, 1999) and exposure to ozone and UV-B. The endogenous SA levels in senescing stage 2 leaves are four times higher than in nonsenescing leaves (Morris et al., 2000). This is consistent with
a role for SA during later stages of the senescence program. Study of the nahG, pad4 and npr1 mutants, which are defective in the SA signaling pathway, showed an altered expression pattern of a number of SAGs. Furthermore, a delay in yellowing and reduced necrosis were observed in these plants (Morris et al., 2000). The pad4 mutant has a non-necrotic phenotype that has a reduced expression of SAG12, a well-known SAG. The authors postulated that SAG12 may take part in a regulatory pathway leading to cell death and that it supports the transition from senescence to final cell death. Thus the senescence phenotype of pad4 mutant suggests that SA might regulate the transition from senescence to final cell death.

Besides biochemical and physiological evidence for role of SA in senescence, genetic evidence has also been generated by a microarray approach (Buchanan-Wollaston et al., 2005). Of 827 genes that were identified as senescence upregulated genes, 19% showed at least a twofold reduction in the nahG transgenic plants that are defective in SA signaling. Interestingly SAG12 expression was substantially reduced compared to that in wild-type plants and SA-treated plants (Morris et al., 2000, Buchanan-Wollaston et al., 2003). Since SAG12 is generally seen as a marker for developmental senescence, this further demonstrates the importance of SA in senescence.

**Involvement of genome programs in the regulation of senescence-associated genes**

Developmental senescence is regulated by diverse programs involved in plant life maintenance, defense responses and growth and development (see above). This is consistent with the evolutionary theory of senescence and the proposal of genome optimization for reproduction, which argues that no specific genetic programs for life span evolve. Since the expression profiles of SAGs are reliable markers for senescence, examining the regulation of SAG expression may provide evidence to support that argument. If the evolutionary theory of ageing applies to plants, we expect that many SAGs encode proteins with functions throughout the life cycle of the plant, and not only during developmental senescence. This notion, as a matter of fact, is well supported by the identities and expression profiles of SAGs. Up to now, almost all the isolated SAGs, including many involved in nutrient salvage, exhibit a certain basal level of expression prior to the onset of leaf senescence. This indicates
that nutrient salvage is a continuous process occurring in plant cells throughout life. In this sense, leaf senescence is not different from other leaf developmental stages but is more committed to recruit the last yet important source of nutrients retained in an ageing leaf.

Recent omics techniques have allowed us to examine the genes that are upregulated during senescence on a whole-genome basis. In addition to development, leaf senescence can be induced by biotic and abiotic stresses. It is therefore possible to compare the SAG expression profiles of various types of senescence using currently available microarray data, which enables the better understanding of the nature of the regulation of developmental senescence. In a whole-genome transcriptome analysis, a total of 827 SAGs were found upregulated during developmental senescence (Buchanan-Wollaston et al., 2005). However, most of those are induced by hormones (SA, JA and ethylene) or darkness as well (Buchanan-Wollaston et al., 2003; Lin and Wu, 2004). Using these data, we deduced the number of SAGs that were enhanced by darkness-induced senescence and were downregulated during leaf senescence in nahG, coi1 and ein2 plants. The remaining SAGs are hence presumably regulated by other developmental cues and/or stress conditions. As shown in Table 1, this category under the name of ‘development’ includes a total of 209 SAGs, which interestingly spread almost in all the categories. We further dissected whether these SAGs are upregulated by carbon and nitrogen metabolism. For this, the array data from ‘Expression patterns of genes induced by sugar accumulation during early leaf senescence’ provided by Wingler’s laboratory were used. Analysis was done by GENEVESTIGATOR (Zimmermann et al., 2004). Nearly half of the 209 SAGs were upregulated after induction of senescence by glucose in combination with low nitrogen. Again, the remaining 110 SAGs are wide spreading in all the categories. If this list of SAGs is compared with the profiles of SAGs in senescence regulated by other developmental cues such as ROS, other hormones (cytokinins, ABA, GA, etc.) or protein degradation, it will not be surprising that perhaps nearly all the SAGs are be regulated by one or more of these cues. In other words, very few SAGs will be solely induced by developmental senescence, which is in agreement with the evolutionary theory of ageing.
Table 1  
Comparison of gene expression profiles of age-regulated and developmental leaf senescence.

<table>
<thead>
<tr>
<th>Category</th>
<th>Total</th>
<th>Dark</th>
<th>Cell suspension</th>
<th>nahG/coi1/ein2</th>
<th>Development</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>C/N Others</td>
</tr>
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<td><strong>Regulatory genes</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Putative transcription factors and nucleic-acid-binding proteins</td>
<td>96</td>
<td>47</td>
<td>38</td>
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<td>9</td>
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<tr>
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<td>3</td>
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<td>1</td>
</tr>
<tr>
<td>Putative ubiquitination control</td>
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<td>23</td>
<td>6</td>
<td>2</td>
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<td>20</td>
<td>31</td>
<td>6</td>
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<td>8</td>
<td>5</td>
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<tr>
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<td>8</td>
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<td>11</td>
<td>6</td>
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<tr>
<td><strong>Macromolecule degradation and mobilization</strong></td>
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<td></td>
<td></td>
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<td></td>
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<td>15</td>
<td>10</td>
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<tr>
<td>Nucleic acid degradation and phosphate mobilization</td>
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<td>8</td>
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<td>Sulfur mobilization</td>
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<td>Carbohydrate metabolism</td>
<td>63</td>
<td>27</td>
<td>22</td>
<td>32</td>
<td>6</td>
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<td>Lignin synthesis</td>
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<td>2</td>
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<tr>
<td>Transport</td>
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<td>4</td>
<td>1</td>
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<td></td>
<td></td>
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<td></td>
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<tr>
<td>Antioxidants</td>
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<td>6</td>
<td>4</td>
<td>2</td>
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<tr>
<td>Stress and detoxification</td>
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<td>4</td>
<td>5</td>
<td>2</td>
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<td>Flavonoid/anthocyanin pathway</td>
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<td>5</td>
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<tr>
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<td>5</td>
<td>3</td>
<td>1</td>
<td>0</td>
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<tr>
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</tr>
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<td>Unknown genes</td>
<td>132</td>
<td>65</td>
<td>55</td>
<td>34</td>
<td>17</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>827</td>
<td>385</td>
<td>335</td>
<td>257</td>
<td>99</td>
</tr>
</tbody>
</table>

Data sources: Buchanan-Wollaston et al. (2005); C/N: carbon and nitrogen supply (Expression patterns of genes induced by sugar accumulation during early leaf senescence; Zimmerman et al., 2004).

The most frequently used SAG to monitor developmental senescence, and perhaps one of the few SAGs which is specifically induced by developmental senescence, is *SAG12*. *SAG12* transcripts were found to be very low or below the detection level in young and mature green leaves, contrasting to the levels of the transcripts of *SAG13* and *SAG14* (Figure 7.1; Lohman et al., 1994). Unlike other SAGs, including *SAG13*, *SEN1* and *SAG14* whose expression could be enhanced in young leaves by a range of senescence-inducing treatments such as detachment, hormonal exposure,
darkness, drought, wounding and pathogen challenge, SAG12 was only occasionally found to change its expression under these circumstances (Oh et al., 1997; Park et al., 1998; Weaver et al., 1998; Noh and Amasino, 1999; Brodersen et al., 2002).

Thus, SAG12 is considered the best marker for developmental senescence that relies on leaf age, whereas SAG13 and SAG14 may represent stress-induced senescence or general cell-death markers. That the SAG12 promoter has been used for the autoregulated production of cytokinin to delay senescence in a number of species including tobacco (Gan and Amasino, 1995; Ori et al., 1999), lettuce (McCabe et al., 2001), petunia (Chang et al., 2003) and Arabidopsis (Huynh et al., 2005) suggests that the developmental senescence regulation of SAG12 is conserved across species. Moreover, a conserved cis-element of the SAG12 promoter was also found in the Asparagus officinalis asparagine synthetase promoter.
and was responsible for the induction of transcription of this gene by senescence (Winichayakul et al., 2004). Thus, monocotyledonous and dicotyledonous plants appear to share this senescence cis-element, further confirming the conservation of the regulation of developmental senescence across species. Extensive studies on the expression of SAGs, including SAG12, have provided exciting new insights into the developmental regulation of senescence, and future research will likely result in a better understanding of developmental senescence.

Integrating hormonal action into developmental senescence

Reproduction has specific timing and all the programs need to be timely in place to ensure successful reproduction. The indirect consequence is that the various strategies embedded in the programs will initiate developmental senescence in an age-dependent manner. Thus, developmental senescence is the consequence of time-specific action of genes. Understanding the timing of the various senescence strategies is a necessary step for elucidating the molecular mechanisms of developmental senescence. In this section we intend to put together the action of the hormones that control leaf senescence and thus developmental ageing in Arabidopsis.

Previously, we proposed a senescence window concept to explain the involvement of ethylene in leaf senescence (Jing et al., 2002, 2003). Depending on whether and how senescence can be induced by ethylene, the life span of a leaf can be split into three distinct phases (Figure 2A). The experimental evidence supporting this view is briefly summarized as follows. (1) When plants were exposed to a short-pulse (e.g. 1–3 days) ethylene treatment, no senescence symptoms could be induced in young leaves (Grbić and Bleecker, 1995; Weaver et al., 1998; Jing et al., 2002). (2) Leaf senescence is not accelerated in the ctr1 mutants (Kieber et al., 1993). This indicated that there exists a never-senescence phase in which senescence cannot be induced by ethylene. (3) Furthermore, in a certain range of leaf ages, the effect of ethylene on leaf senescence increases with the increase in leaf age (Grbić and Bleecker, 1995; Weaver et al., 1998; Jing et al., 2002), indicative of an ethylene-dependent senescence phase. (4) Finally, beyond certain leaf ages, senescence will start even without the participation of ethylene as shown in the etr1 and ein2 mutants in which
Figure 2. The senescence window concept. (A) The senescence window concept as deduced from the effects of ethylene on leaf senescence. At early leaf development, ethylene is not able to induce leaf senescence. This is the so-called never-senescence phase in the model. Only after a certain developmental stage, ethylene can induce leaf senescence, depending on the environmental conditions. Further development of the leaf will always result in senescence, even in the absence of ethylene. (B) Hormonal action during leaf development is age dependent. The action of the senescence-promoting hormones is strongest at late developmental stages, and is antagonistic to that of the stay-green hormones. The onset of leaf senescence is achieved by a depletion of the stay-green hormones, concomitant with an increase in ethylene levels followed by JA, ABA and eventually SA. Hormone levels and sensitivity change during development, as indicated by the triangles. The age-related changes limit the action of the various plant hormones to their own specific age window.

the senescence progresses normally once started (Griči and Bleecker, 1995; Park et al., 1998; Buchanan-Wollaston et al., 2005), which suggests the existence of an ethylene-independent senescence phase. This senescence window concept emphasizes the developmental control of leaf senescence and considers leaf age as
an ultimate determinant of senescence progression. Clearly, genes that control the phase transitions of the senescence window are important for the onset of developmental senescence, and evidence suggests that many genetic loci are required (Jing et al., 2002, 2005). Thus, the senescence window concept provides an explanation why the senescence-promoting effect of ethylene is variable during development.

The senescence window concept can, perhaps, integrate the action of all plant hormones involved in leaf senescence. In *Arabidopsis* the different hormones seem to control the onset and progression of senescence in an age-related manner. Figure 2B is an extension of the senescence window concept developed from the interaction between leaf age and ethylene and shows a tentative model illustrating the timing and action of the different hormones during developmental senescence. In this model, age-related changes, and thus development, are considered the primary regulator of leaf senescence. During ageing, developmental cues lead to the diminished action of the senescence-retarding hormones such as auxin, GA and cytokinins, as well as the concomitant strengthening of the action of senescence enhancing hormones such as ethylene, JA, ABA and SA. The action of the different hormones during the initiation of leaf senescence does not change suddenly but gradually, allowing a gradual integration of all the hormones controlling the process. This suggests that the senescence process is partly reversible by fine-tuning hormone action and hence amenable for modulation.

The model provides a basis for the explanation of experimental data. For instance, the major senescence-retarding compound cytokinin can delay senescence when its level is maintained. However, in transgenic *SAG12–IPT* plants the senescence process will start eventually and progresses normally (Gan and Amasino, 1995; Ori et al., 1999), suggesting that cytokinin action is restricted to certain developmental stages. On the other hand, cytokinin biosynthesis mutants showed a shorter leaf life span (Masferrer et al., 2002). This might be explained by assuming that the effect of the senescence-promoting hormones is antagonistic to those blocking senescence; older leaves may become less sensitive for cytokinin and more sensitive for senescence-promoting hormones like JA and ABA (Weaver et al., 1998). Similarly, blocking the ethylene pathway increases leaf longevity. Finally, however, the leaves go into senescence because the influence of JA, ABA and SA may increase with the
age of the leaf. Thus, the age-related changes limit the action of the various hormones to their own specific window.

Taken together, although plant hormones are almost universally involved in every aspect of plant life, they may participate into developmental senescence only in very specific age windows. The proposed senescence window concept and the model for hormonal action provide a developmental view to examine the modulation of developmental senescence by hormones, which certainly requires more experimental evidence for validation.

**Outlook and perspectives**

Thanks to the availability of cutting-edge technology and the use of model species with known whole-genome sequences that have enabled senescence studies to be carried out at a scale that was not imaginable even 15 years ago, our knowledge on the regulation of developmental senescence has been advanced tremendously. It is clear that hormonal modulation, metabolic flux, ROS and protein degradation are the major cellular and molecular processes that are important for senescence regulation. Strikingly, these processes are embedded in the genome programs that regulate plant life maintenance, responses to biotic and abiotic stresses, and growth and development for the sake of successful reproduction. Thus, leaf senescence can be viewed as an indirect consequence of genome optimization for reproduction. This perspective is exciting and worthy of further exploitation, since it coincides with the evolutionary theory of senescence developed from animal ageing studies. In-depth molecular genetic studies are required to dress the evolutionary basis of leaf senescence. In particular, identification of regulatory genes with pleiotropic functions or late-life deleterious effects should be a priority for further senescence studies.

The complexity of leaf senescence is mainly due to the involvement of multiple components that exhibit overlapping effects. This is particularly true for the action of hormones. The proposed senescence window concept provides a theoretic framework to dissect the action of hormones during senescence depending on their time of action, which is important to separate the effect of hormones on senescence from their other effects. Using this concept, it is possible to study genetic components that control the action of hormones during development, which is an essential step for ultimately understanding the mode of action of hormones during development.
Combined with the genetic dissection, whole-genome analysis should be employed to define the networking of various regulatory circuits.

In conclusion, senescence is one of the biological phenomena with extreme complexity. In the current postgenome era, we are provided with both opportunities and the challenge to dissect the molecular genetic mechanisms of leaf senescence. The findings in the past have enabled us to look at senescence regulation from a fresh perspective of genome optimization. We have evolutionary and developmental theories that guard us to define the proper targets. We are also armed with cutting-edge technologies and tools. Thus, a concerted effort will eventually unveil the mystery of senescence regulation and provide a genetic basis for senescence manipulation.
References


