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

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

Keywords: leaf senescence, old mutants, ageing, ethylene, senescence-associated genes, Arabidopsis

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

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

Various factors influence the onset of leaf senescence. Leaf senescence is an intrinsic age-dependent process, and its onset appears to be regulated by the age of individual leaves (Hensel et al., 1993). Senescence is also prematurely induced by a range of external factors such as darkness,
detachment, drought and pathogen attack (Park et al., 1998; Pontier et al., 1999; Quirino et al., 1999; Weaver et al., 1998). Under these induction conditions distinct sets of SAGs are induced, suggesting the existence of a complex regulatory network. The complexity is further shown by a recent screening for SAGs which takes advantage of an enhancer trap system (He et al., 2001). In addition, plant hormones have been implicated in regulating leaf senescence (Gan and Amasino, 1995; He et al., 2002; Morris et al., 2000).

The role of the volatile phytohormone ethylene in leaf senescence is revealed by studies on ethylene-treated plants and ethylene mutants as well as on transgenic plants. On the one hand, ethylene promotes leaf senescence. In wild-type Arabidopsis plants, ethylene treatment can advance the visible yellowing and SAG induction in leaves that are primed to senesce (Grbic and Bleecker, 1995; Weaver et al., 1998). Arabidopsis ethylene-insensitive mutants, exemplified by ctr1, ein2 and ein3, display delayed leaf senescence (Bleecker et al., 1988; Chao et al., 1997; Grbic and Bleecker, 1995; Hua and Meyerowitz, 1998; Oh et al., 1997). On the other hand, ethylene is neither necessary nor sufficient for the occurrence of senescence. Senescence eventually occurs in the ethylene insensitive mutants. Ethylene constitutive response and overproduction mutants such as ctr and eto do not show premature leaf senescence as could be expected (Guzman and Ecker, 1990; Kieber et al., 1993). In tomato plants carrying the Never ripe mutation or with the antisense suppression of the ACC oxidase, the progress of leaf senescence is not retarded once senescence has started (John et al., 1995; Lanahan et al., 1994). Together, these studies suggest that ethylene does not directly regulate the onset of leaf senescence. It acts to modulate the timing of leaf senescence.

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

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

Results

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

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

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

The above experiments provided evidence for the following notions: (i) Arabidopsis cotyledons have to reach a defined developmental stage to perceive ethylene’s action in inducing senescence; (ii) An ethylene-treated mutagenised plant that shows yellow cotyledons before 17 days or no yellow symptoms after 24 days, can be considered a putative early or late leaf senescence mutant, respectively. These notions led to the establishment of a screen system for isolating leaf senescence mutants. Both early and late leaf senescence mutants were isolated as described in Experimental procedures and we named them old (onset of leaf death) mutants. In this paper, we focus on early leaf senescence mutants and report the characterisation of mutations in 3 genetic loci that are implicated in the regulation of leaf senescence.

Early leaf senescence mutants fall into 3 complementation groups

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

The old1 locus is represented by 3 recessive alleles isolated from independent M2 pools. We selected old1-1 for future studies. The old1-1 plants have a strong early leaf senescence phenotype. After approximately 14 days of growth, old1-1 cotyledons became yellow, while wild-type cotyledons were still green (Figure 2a,b). By 24 days yellowing was observed in the first 2 rosette leaves as well, and ethylene treatment strongly promoted leaf senescence in old1-1 plants (Figure 2g,h). The adult old1-1 plants start bolting and flowering earlier (bolts arose in old1-1 with 4–7 rosette leaves and in Ler-0 with 8–9 rosette leaves) and had a reduced size and less progeny. The old1-1 mutation was mapped to the bottom of chromosome 5, approximately 3 centiMorgans south of the single nucleotide polymorphism (SNP) marker SGCSNP84.

The old2 plants are indistinguishable from wild-type plants in air (Figure 2e,i), but ethylene triggers accelerated senescence. After ethylene treatment at 21–24 days, old2 plants showed yellow cotyledons and first 2 rosette leaves, whilst wild-type plants had yellow cotyledons (Figure 2f,j). Genetic analysis revealed that old2 is an ethylene-required dominant trait and that there is a dominant suppressor of
old3 in Col-0 (Table 1). These characteristics make mapping a rather time-consuming process. The old2 mutant belongs to Class II early leaf senescence mutants, and among 12 mutants isolated 6 showed similar phenotypes and complex genetic behaviour, including the presence of a dominant suppressor in Col-0. It seems likely that they represent the same locus, but this needs to be confirmed by further genetic analysis.

One single old3 allele was identified. When grown in soil, the homozygous old3 mutant is rosette-lethal and plants stop growing when the second pair of rosette leaves appears (Figure 2c). Interestingly, old3 can be rescued by culture on medium with 1% sucrose, although the plants still senesce earlier (Figure 2d). Ethylene treatment enhanced the speed of senescence in cotyledons but did not induce yellowing in additional leaves. A heterozygous plant with intermediate old3 phenotypes was backcrossed to the parental line Ler-0, and the progeny tests showed that old3 is a semi-dominant allele (Table 1). In all the F1 plants of an old3 heterozygote by Col-0 cross, the wild-type phenotype was observed indicating that a suppressor exists in Col-0. Subsequent observations of 39 independent F2 populations showed that 20 of them contained the old3 mutation and the segregation ratio manifested a duplication of a semi-dominant gene in Col-0. Plants homozygous for both the suppressor and the old3 loci were used for mapping, and the two loci were placed at the lower arm of chromosome 4, approximately 3.6 centiMorgans north of CAPS marker G4539a.

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

The senescence syndrome is advanced in old mutants

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

To examine the physiological changes during senescence, the chlorophyll content and the ion leakage were measured in whole rosette, cotyledons and different rosette leaves from 11-day, 16-day, 24-day, 30-day and 40-day-old plants. Results for the 24-day samples are presented in Figure 3. In the whole rosette samples isolated from air-grown seedlings, a lower chlorophyll content was found in old1 and old3, while a higher ion leakage was only observed in old3, but not in the old1-1 samples. No differences were found between old2 and the wild-type. Upon ethylene treatment, samples of the old plants showed lower

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### Table 1 Genetic segregation of early leaf senescence mutants

<table>
<thead>
<tr>
<th>Male</th>
<th>Female</th>
<th>Generation</th>
<th>Wild-type</th>
<th>Mutant</th>
<th>$\chi^2$</th>
<th>P</th>
</tr>
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<td>old1-1 Ler-0</td>
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<td>17</td>
<td>0</td>
<td>1.059</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>old1-1 Ler-0</td>
<td>F1</td>
<td>0</td>
<td>29</td>
<td>1.059</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>old1-1 Ler-0</td>
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<td>0</td>
<td>22</td>
<td>1.059</td>
<td>0.29</td>
<td></td>
</tr>
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<td>old2 Ler-0</td>
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<td>0</td>
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<td>2.910</td>
<td>0.08</td>
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<td>237</td>
<td>1.469</td>
<td>0.22</td>
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<td>old3 Ler-0</td>
<td>F1</td>
<td>6</td>
<td>7</td>
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<td>0.79</td>
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<tr>
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<td>F1</td>
<td>140</td>
<td>0</td>
<td>0.136</td>
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<td>F2</td>
<td>355</td>
<td>120</td>
<td>0.08</td>
<td>&gt;0.29</td>
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</tbody>
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a The old2 heterozygotes had phenotypes similar to that of the parental old2 homozygote. The genotype scoring was carried out on 24-day-old plants treated with 10 μl l⁻¹ ethylene for 3 days. The criteria were: wild-type plants with 0–3 yellow cotyledons and/or leaves, old2 plants with 2 yellow cotyledons and at least 2 yellow leaves.

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

c The pollen from old3 heterozygotes was used for pollination since the old3 homozygotes were lethal. Two types of F1 plants were obtained in the backcrosses to Ler-0, whereas in the backcrosses to Col-0 all the F1 progeny showed the wild-type phenotypes.

d The progeny of the OLD3/old3 F1 plants were used for segregation test. These numbers represent heterozygous/homozygous old3.

chlorophyll content and higher ion leakage compared to the wild-type. These results were consistent with the visible yellowing of the mutants and the effect of ethylene in inducing visible yellowing (Figure 2), suggesting an overall acceleration of leaf senescence in the old mutants.

More detailed analyses using cotyledons, the rosette leaves 1/2, 3 and 4 also showed the advanced leaf senescence in the old mutants. For air-grown samples, the chlorophyll content and ion leakage of old1-1 and old3 were significantly different from those of old2 and the wild-type, except the rosette leaf 4 from old1-1. Upon ethylene treatment, the chlorophyll content of cotyledons dropped to the minimal for all the plants and no differences were observed, but the ion leakage of the old mutants was still higher than that of wild-types. In ethylene-treated rosette leaves 1/2 large differences were observed between old mutants and the wild-type for both parameters, but in rosette leaves 3 and 4 the old mutants differed from the wild-type in the chlorophyll content, not in the ion leakage, further showing the sequential occurrence of chlorophyll degradation and membrane damage. Together, lower chlorophyll contents and higher amounts of ion leakage were observed in all mutant samples except the air-grown old2 and the younger the leaf, the less the differences between the old mutants and the wild-type, indicating that leaf senescence in the old mutants occurred earlier and was controlled by leaf age. These results also suggested that although the mutants were isolated on the basis of the altered senescence phenotypes of cotyledons, the senescence of the rosette leaves was similarly affected.

We subsequently performed a detailed molecular characterisation by Northern blot analysis of the mRNA abundance of the genes listed in Table 2. Changes in the mRNA levels of the tested genes fell into distinct classes (Figure 4). The first class is represented by CAB whose mRNA levels reduce during senescence. The levels of CAB mRNA were already lower in air-grown old2-plants and more strongly reduced upon ethylene treatment. The second class includes SAG13, SAG14 and SAG21. In air, the mRNA levels of these genes in old1 and old3 were higher than those in old2 and the wild-type. Upon ethylene treatment, more

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

Figure 6. Phenotypes of old1-1, etr1-1 and old1-1etr1-1 mutants. Seedlings were grown in the conditions described in Experimental procedures for 17 days and photographed. Bar represents 1 cm.
Figure 3. Comparison of chlorophyll content and ion leakage between the wild type and the three old mutants. Ler-0 plants and old mutants were grown either in air for 24 days, or first in air until 21 days and then exposed to 10 μl l⁻¹ ethylene for 3 days, and the whole rosette or leaves at specific positions were isolated and the chlorophyll content and the ion leakage quantified. Results are shown as mean ± SE of at least three replicates. Note the chlorophyll content results between ethylene treatment and air were not comparable due to the inhibitory effect of ethylene on cell expansion.

Table 2. Genes used for hybridisation experiments

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Encoded protein</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>CAB</td>
<td>Chlorophyll a/b binding protein</td>
<td>Leutwiler et al. (1986)</td>
</tr>
<tr>
<td>PR-1</td>
<td>Acidic pathogen-related protein</td>
<td>Uknes et al. (1992)</td>
</tr>
<tr>
<td>SAG2</td>
<td>Cysteine protease</td>
<td>Hensel et al. (1993)</td>
</tr>
<tr>
<td>SAG12</td>
<td>Cysteine protease</td>
<td>Lohman et al. (1994)</td>
</tr>
<tr>
<td>SAG13</td>
<td>Short chain alcohol dehydrogenase</td>
<td>Lohman et al. (1994)</td>
</tr>
<tr>
<td>SAG14</td>
<td>Blue copper-binding protein</td>
<td>Lohman et al. (1994); Weaver et al. (1998)</td>
</tr>
<tr>
<td>SAG21</td>
<td>LEA protein</td>
<td>Weaver et al. (1998)</td>
</tr>
</tbody>
</table>
pronounced increases in the mRNA levels of these SAGs were observed in the *old* mutants in comparison with the wild-type. The third class is shown by SAG2, which showed a basal mRNA level that was higher in air-grown *old1* and *old3* plants, consistent with the early occurrence of senescence in these two *old* mutants. Upon ethylene treatment, the SAG2 mRNA levels did not appear to be higher in *old* mutants, although the mutants showed stronger senescence. The mRNA level of SAG2 is also increased by exogenous ethylene in green leaves besides being up-regulated during senescence (Grbic and Bleecker, 1995; Hensel *et al*., 1993), which may explain why in ethylene-treated samples little difference was detected between *old* mutants and the wild-type plants.

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

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

The particular phenotype of *old2* mutants prompted us to perform more detailed gene expression analysis on ethylene-treated leaves. The 21-day *old2* and wild-type plants were exposed to ethylene for 3 days, and the CAB and SAG mRNA levels from the second and third rosette leaves were analysed. In this experiment, over 95% of the second rosette leaves were still green in the wild-type after ethylene treatment, whereas over 90% showed signs of senescence in the *old2* plants. For the third rosette leaves only the green ones were used for both *old2* and the wild-type, although approximately 50% of the *old2* third rosette leaves were yellow. Compared to the wild-type controls, *old2* leaves had lower levels of CAB mRNA and higher mRNA levels of the tested SAGs. Crucially, even in the green third rosette leaves, the mRNA levels of the tested genes showed large differences between *old2* and the wild-type. In particular, the mRNA levels of SAG12 and SAG13, which are believed to be good markers for natural leaf senescence (Weaver *et al*., 1998), were prominent in *old2* but undetectable in the wild-type. This comparison more

<table>
<thead>
<tr>
<th>Gene</th>
<th><em>old1</em></th>
<th><em>old2</em></th>
<th><em>old3</em></th>
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<td>Low</td>
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<tr>
<td>SAG21</td>
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<td>SAG2</td>
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<td>SAG14</td>
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</table>

Figure 4. Northern hybridisation analysis showing the abundance of the mRNA of various genes in *old* mutants and wild-type plants. The left column shows RNA samples from the whole rosette of 24-day *old1-1*, *old2* and the wild-type (Ler-0) plants. The middle column shows RNA samples from the whole rosette of 16-day and 24-day *old3* and the wild-type plants. The right column shows RNA samples from rosette leaf number 2 the green leaf number 3 of the 24-day *old2* and wild-type plants after a 3-day ethylene treatment. For samples in the left and the middle columns 10 μg total RNA was loaded per lane, and for samples in the right column 5 μg RNA was loaded. The sources for each gene are shown in Table 2.
old mutants exhibit different ethylene responses

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

The old mutants may work in a common regulatory pathway

Since the three old mutants showed early senescence phenotypes and similar SAG expression profiles, they may work in a common regulatory pathway controlling leaf senescence. We performed double mutant analysis to determine the potential genetic interactions between the OLD genes (Table 3). When old1-1 was crossed to old2, the plants with old1-1 phenotypes segregated in a 1:3 ratio; while in the F2 plants of an old1-1 by old3 cross plants showing old1-1 and old3 phenotypes segregated in a 3:4 ratio, which suggests that OLD1 might work upstream of OLD3 and downstream of OLD2. Like old3 the old1-1old3 double mutant is also rosette-lethal. The results from testing the relationship between old2 and old3 were also consistent with putting OLD3 downstream of OLD2. Overall, results of the epistatic analysis correlated well with the phenotypes of the mutants and the results from SAG expression analysis, implying that the OLD genes may control a common regulatory pathway for leaf senescence.

Owing to the particular phenotypes of old1-1, we considered that OLD1 may act at the linking point between ethylene- and age-regulated leaf senescence and constructed the double mutant of old1-1 and etr1-1. As shown in Figure 6, at 17 days the air grown old1-1etr1-1 showed senescing cotyledons as in the old1-1, suggesting that the earlier onset of leaf senescence in old1-1 is independent of ethylene perception. On the other hand, when treated with

Table 3 Segregation analysis of F2 progeny of crosses among three old mutants

<table>
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<tr>
<th>Crosses</th>
<th>WT</th>
<th>old1-1</th>
<th>old2</th>
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<td>87</td>
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<td>–</td>
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<tr>
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<td>102b</td>
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<td>148</td>
<td>80</td>
<td>3.04</td>
<td>&gt;0.38</td>
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</table>

Phenotype scoring was detailed in Experimental procedures. WT: wild type; He: heterozygous; H: homozygous

a A 3:4:9 ratio of WT:old1-1:old2 was tested.
b old1-1old1-1/OLD3old3 and old1-1old1-1/OLD3OLD3 showed old1-1 phenotypes.
c A 3:3:6:4 ratio of WT:old1-1:He-old3:H-old3 was tested.
d A 1:3:8:4 ratio of WT:old2:He-old3:H-old3 was tested.
ethylene, the double mutant failed to show a more exaggerated senescence syndrome as in the old1-1 indicating that the progression of leaf senescence in old1-1 does require a functional ethylene pathway. Taken together, these results suggest that OLD1 mediates the actions of ethylene and age-related factors in the regulation of leaf senescence.

Discussion

The senescence window

In an attempt to identify genetic loci that define the onset of leaf senescence, the interaction between ethylene and leaf age was studied in Arabidopsis. The effect of ethylene in inducing senescence varied largely, depending on the time when it was applied. Our results further confirmed the notion that ethylene can only induce senescence after a leaf reaches a certain developmental stage (Grbic and Bleecker, 1995; Weaver et al., 1998). In extreme situations such as in the ctr plants in which ethylene signalling is constantly switched on, or in wild-type plants that are grown in the continuous presence of exogenous ethylene, early leaf senescence is not induced (data not shown, Kieber et al., 1993). In opposite situations such as in the ein mutants in which the ethylene signalling is permanently blocked, or in the plants with antisense constructs that reduce ethylene biosynthesis, normal leaf senescence eventually occurs (Alonso et al., 1999; Bleecker et al., 1988; Chao et al., 1997; Grbic and Bleecker, 1995; John et al., 1995; Oh et al., 1997). These observations exhibit that ethylene has no effect in stimulating senescence before or after certain developmental stages. In conclusion, a leaf has a defined age window to perceive the effect of ethylene on senescence, which is termed here as the senescence window.

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

Many genes are involved in the biosynthesis, signalling and action of ethylene and are differentially regulated. In Arabidopsis, 5 genes encoding ACC synthase are differentially expressed in plant organs, during development and in response to various stress conditions (Liang et al., 1992). Throughout plant development, the expression of the ETR ethylene perception gene family is differentially regulated in tomato (Lashbrook et al., 1998). In both Arabidopsis and tomato a large gene family of EIN3 and EIL was identified (Chao et al., 1997; Tieman et al., 2001). Downstream are ethylene-response-factor1 and ethylene-responsive element binding factors whose differential regulation is also observed (Fujimoto et al., 2000; Solano et al., 1998). Such a sophisticated biosynthesis and signalling pathway is consistent with the existence of the diverse ethylene response windows.

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

Functions of OLD genes

Bearing the model in mind, we tried to select those mutants in our collection with various senescence phenotypes that might locate at different positions in the regulating pathway. The phenotypes of the old1, old2 and old3 suggested that they potentially represented genes acting at the proposed positions (Figure 7). The physiological and molecular

![Figure 7. A genetic pathway showing the proposed positions of the OLD genes in the regulation of leaf senescence.](image)
analyses were consistent with the suggested positions of the OLD genes, which were further confirmed by genetic interaction analysis.

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

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

It is important to notice that the old1 mutants were different from ctr and eto that show a constitutive ethylene response (Guzmán and Ecker, 1990; Kieber et al., 1993). OLD1 is likely to define a novel link integrating ethylene actions into leaf senescence. Ethylene is shown to interact with many other signalling molecules such as with ABA in seed germination (Beaudoin et al., 2000), with jasmonic acid in conferring defence response (Penninckx et al., 1996), with glucose in regulating seedling growth (Zhou et al., 1998), or with light in controlling hypocotyl elongation (Smalle et al., 1997). Isolation and analysis of OLD1 may provide important insights on the interaction between senescence and ethylene signalling pathways.

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

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

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

In summary, we have presented a model in an attempt to explain the control of senescence by age-related factors, the integration of ethylene action, and the regulation of
SAG expression during the execution process. In contrast to the wealth of knowledge on the ethylene signalling pathway, our current information on the molecular mechanisms of the regulation of leaf senescence and on the involvement of ethylene in senescence is scarce. Our model, although rudimentary, matches well the current knowledge available. The phenotypic, genetic and SAG expression analysis of the mutants provided information concerning at which steps the genes act and allowed for the construction of the model. Further refinements, through the isolation of mutants in additional steps, the analysis on double mutants from the OLD and the ethylene signalling pathways, and cloning and characterisation of the OLD genes, should yield more elaborate models for the regulation of leaf senescence.

Experimental procedures

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

Seed mutagenesis and isolation of mutants
Approximately, 20,000 seeds were treated with 0.3% ethyl methanesulphonate (EMS) for 15 h as previously described by Dijkwel et al. (1997). M₁ (mutagenised generation fl) seeds were sown onto 500 pots of 20 cm in diameter, and seeds from individual pots were separately harvested and formed independent pools.

For each M₂ pool approximately 300 seeds were sown on a 20 × 30 cm tray. M₂ plants were grown for 11 days in air, treated with 10% 1⁻¹ ethylene (AGA, The Netherlands) for 3 days, and plants with yellowing cotyledons selected. The remaining plants were allowed to grow further in air for 7 days and treated with ethylene between 21 and 24 days, and plants with no signs of yellowing were sought. In total, approximately 150,000 plants were screened, and approximately 700 putative mutants were isolated. M₂ plants were further re-screened twice to eliminate false positives. Eventually, 62 mutants from 54 pools were obtained. The mutant lines were crossed twice to Ler-0 and etr1-1. The F₂ progeny of the old2 × old3 cross.

To generate double mutants of old1-1 and etr1-1, the old1-1 pollen was used. The F₂ population was grown in soil and seedlings showing old1-1 phenotypes were selected to check the presence of the etr1-1 mutation using a PCR marker (Hua and Meyerowitz, 1998). Since old1-1 was isolated from Ler-0 and etr1-1 was from Col-0, we also used the SNPs to genotype old1-1etr1-1. The F₃ progeny was used for experiments.

Measurements of chlorophyll content, ion leakage and visible yellowing
Individual leaves or whole rosettes were taken from at least 6 seedlings for chlorophyll content and ion leakage measurements. Chlorophyll was extracted in 80% (v/v) acetone overnight at 4°C in darkness and quantified spectrophotometrically using the method of Inskoop and Bloom (1985). For measuring ion leakage, leaf samples were immersed into deionised carbonate-free water, shaken in a 25°C water bath for 30 min, and the conductivity was measured using a Wissenschaftlich Technische Werkstatten conductivity meter (model KLE1/T, Weilheim, Germany). Samples were boiled for 10 min and conductivity measured again. The percentage of the first measurement over the second measurement was used as the membrane damage indicator. Cotyledons with over 10% yellow area of the blade were judged as yellow, corresponding to S1-S5 stages as described by Lohman et al. (1994). After prolonged growth in air, cotyledons showed some degreening, which was not judged as senescence as long as no concomitant yellowing was observed.

RNA extraction and Northern blot analysis
Plant material was frozen in liquid nitrogen and total RNA was isolated and quantified spectrophotometrically. For RNA blot analysis, 5 or 10μg of RNA samples were separated by electrophoresis on a 1.2% agarose gel containing 1.8% formaldehyde and then capillary-blotted onto Hybond-N⁺ nylon membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Ethidium bromide was included in the sample-loading buffer at a concentration of 4μg ml⁻¹ to confirm equal sample loading and blotting. Probes described in Table 2 were 3²P-labelled by random-priming, and hybridisation was done at 65°C overnight in the Church and Gilbert (1984) buffer, and the membranes were washed once in 2X SSC for 5 min and twice for 15 min each in 0.1X SSC and 0.1% SDS at 65°C. Probe hybridisation was visualised with a phosphorimager using
the OptiQuant software (Canberra Packard, Zelik, Belgium). Stripping of the membranes was done by boiling in 0.5% SDS solution for 3 min. Each membrane was stripped and re-probed up to three times.

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