

Identification of *Arabidopsis* stay-green mutants with a functional ethylene-response pathway

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Abstract

Natural or harvest-induced senescence is a major determinant factor causing crop losses. The plant hormone ethylene is a strong inducer of senescence and decreasing the ethylene response can reduce senescence, albeit often with undesirable pleiotropic effects. We took advantage of ethylene-induced leaf senescence as a tool to screen for late senescence *Arabidopsis* mutants that still have a functional ethylene-signalling pathway. Sixteen *Arabidopsis* *onset of leaf death (old)* mutants were selected that stayed green after treatment with ethylene. While all the mutants responded to ethylene in a triple response assay, ten mutants responded to the treatment in the same way as the wild type. These ten mutants showed limited pleiotropic effects when grown under standard growth conditions but nine mutants flowered slightly later than the wild type. Genetic characterisation of a subset of the mutants identified several independent loci controlling the leaf senescence process. The approach resulted in the isolation of several stay-green mutants with a functional ethylene response pathway. The late senescence mutants show extended leaf longevity and further research may advance the field of pre- or post-harvest senescence technology. The results, moreover, suggest that there is a correlation between senescence and floral induction.

Keywords: Senescence, *Arabidopsis*, ethylene, mutant, shelf life

Introduction

Senescence in higher plants is a developmental phase that is manifested by a distinctive decline in photosynthetic activity in association with a visible change in leaf colour (Lim *et al.* 2007). During leaf senescence, the sum of morphological, physiological, and biochemical changes are generally referred to as the senescence syndrome. This comprises dismantling of cell structures starting with chloroplast and hydrolysis of macromolecules such as RNA, DNA, lipids and proteins (Buchanan-Wollaston *et al.* 2003).

Leaf senescence can contribute to pre- and post-harvest losses in vegetable, horticultural and cereal crop plants (Buchanan-Wollaston *et al.* 2003; Lim & Nam 2005). Thus identification and subsequent modification of key regulatory pathways involved in the induction of the leaf senescence process can contribute towards reduction of senescence-induced harvest losses. The analysis of genetic variants that show an increased life span can successfully identify regulatory pathways. Plant varieties with a phenotype that stay-green longer (so-called 'stay-green' phenotypes or mutants) occur naturally among many plant species (Thomas & Smart 1993). In addition, stay-green mutants have been reported in *Arabidopsis thaliana* and crop plants (Walulu *et al.* 1994; Fang *et al.* 1998; Spano *et al.* 2003; Lim & Nam 2005). Altering hormone biosynthesis or response pathways has been successful in delaying the senescence process as well.

Ethylene is a well-documented key hormone in the regulation of the onset and hastening of leaf senescence as well as fruit ripening and flower senescence (Abeles *et al.* 1988; Zacarias & Reid 1990; Grbić & Bleecker 1995; Jing *et al.* 2002). Indeed, most mutants that have a defect in ethylene biosynthesis or signalling show a delayed senescence response. Ethylene-insensitive mutants *etr1* and *ein2* and tomato plants carrying an antisense construct against the ethylene biosynthesis enzyme ACC oxidase showed enhanced leaf longevity (John *et al.* 1995; Oh *et al.* 1997), demonstrating the importance of ethylene as a positive regulator of senescence. However, next to its role in the regulation of senescence, ethylene is implicated in regulating a wide range of developmental processes from seed germination to advanced plant growth stages, cell expansion, flower development, as well as environmentally-induced stress responses and disease resistance (Johnson & Ecker 1998). Therefore plants that carry mutations in the ethylene biosynthesis or response pathway often display pleiotropic phenotypes. For example, ethylene-insensitive tobacco plants lack non-host resistance against soil-borne fungi (Knoester *et al.* 1998), while *Arabidopsis ein2* mutants showed more sensitivity to certain pathogens (Thomma *et al.* 1999). Thus although altering ethylene responses can result in delayed senescence, pleiotropic effects may limit the usefulness of this approach in agriculture.

In this study, we aimed to isolate stay-green mutants that show a minimum of pleiotropic phenotypes. Since ethylene plays an important role in the induction of natural and stress-induced senescence, we intended to isolate mutants that show reduced senescence symptoms after ethylene-induced senescence, but which still have a functioning ethylene response pathway. Such mutants may have a mutation that more specifically affects the senescence syndrome exclusively and are likely to lack

pleiotropic phenotypes that are typically present in ethylene response mutants. We used ethyl methanesulfonate (EMS)-mutagenised *Arabidopsis thaliana* seeds to screen for mutants (Jing *et al.* 2002). The availability of extensive genomic resources for *Arabidopsis* makes it a very attractive model plant for the identification and functional analysis of senescence-regulated genes. The mutant screening resulted in identification of 16 stay-green mutants that show a significant extension in leaf longevity but with a functioning ethylene response pathway. The molecular mechanisms regulating the leaf longevity remain poorly understood and we anticipate that the knowledge gained with the future analysis of the mutants will prove useful for the further development of crops and horticultural plants.

Methods

Plant materials and growth conditions

Arabidopsis thaliana, accession Landsberg *erecta* (Ler-0) was the parental line for the *onset of leaf death (old)* EMS-mutagenised mutants. Wild type plants were grown continuously in air for different amounts of time and were subsequently grown either for four days in air, or for three days in air supplemented with a dosage of approximately 10-40 µl/l ethylene, followed by one day of growth in air. Ler-0 wild type seed was EMS-mutagenised and putative mutants were isolated as reported by Jing *et al.* (2002). The progeny of approximately 300 putative mutants were treated for 26 days with air, followed with three days of air supplemented with ethylene and one additional day with air. The number of yellow leaves was subsequently scored. The triple response assay was performed as described by Jing *et al.* (2005). For phenotypic analysis, more than 25 of each of the mutants were grown for 30 days and compared to wild type Ler-0 plants. At 30 days, the floral buds were visible on >90% of the wild type plants. Mutants in which the

floral buds of <25% of the plants were visible, were considered to be late flowering. Noticeable differences between the wild type and the mutants were visually scored.

Genetic characterisation

The mutants were crossed to the wild type *Ler-0* for the determination of the genetic behaviour. For mapping, the *old* mutants were crossed to *Col-0*. The F2 seedlings were grown for 26 days in air, treated for 3 days with ethylene and subsequently grown for one additional day in air. At least 100 F2 plants with a late senescence phenotype were selected for DNA isolation. DNA was isolated and genetic linkage was analysed as described (Jing *et al.* 2007).

Results

Response of wild type *Arabidopsis* plants to ethylene-induced senescence

Ethylene is a strong inducer of senescence in *Arabidopsis* (Abeles *et al.* 1988; Zacarias & Reid 1990; Jing *et al.* 2002). First we measured the response of wild type *Arabidopsis* plants to ethylene-induced senescence. Figure 1 shows that plants grown for 35 days in air show visible leaf yellowing in some of the cotyledons, while after 42 days in air approximately five leaves were yellow. Ethylene treated wild type plants responded to ethylene by showing cotyledon yellowing after just 25 days of growth, and this number increased to more than ten leaves in 42-day-old plants. No yellow leaves were observed in wild type plants grown in air for 30 days, while plants treated with ethylene showed an average of six yellow leaves. The results confirm that ethylene is a strong inducer of leaf senescence and that its effect depends on the age of individual leaves.

Selection of late senescence mutants

Previously we isolated approximately 300 putative *Arabidopsis* late senescence mutants (Jing *et al.* 2002). Many of those were expected to be false positives and

needed to be re-screened. The progeny of the putative mutants were treated with ethylene and the senescence phenotype was determined after 30 days of growth. This treatment resulted in an average of 6 yellow leaves in the wild type (Figure 1) and putative mutants that had a maximum average of four yellow leaves were selected as true mutants. The secondary screen identified seventeen true mutants that showed between zero and four yellow leaves (Figure 2). The mutants were called *old101* to *old116* and *old118*. The mutations cause a late senescence phenotype, suggesting that the mutated genes are involved in the stimulation of (ethylene-induced) senescence. The genetic behaviour of 15 mutants was analysed by backcrossing the mutants to the wild type *Ler-0* accession. The F1 and F2 phenotype of several mutants was subsequently determined, and the *old101-1*, *old102*, *old104* and *old106* mutations segregated as monogenic recessive traits (Table 1). For the other mutants only the F1 was tested. The data suggest that dominant, co-dominant and recessive mutations are present in the mutant collection. Five mutants were selected for mapping. The *old101*, *old102*, *old103*, *old104* and *old106* mutants were crossed to the *Arabidopsis* accession *Col-0*. Late senescence phenotypes from a segregating F2 population were subsequently isolated and the map position of these *OLD* genes was subsequently estimated as shown in Table 1. The *old101* mutation segregated as a monogenic recessive trait when backcrossed to its wild type accession *Ler-0*. However, analysis of the F2 population of a cross between *old101* plants and *Col-0* revealed that the *old101* phenotype did not segregate as a single locus. Instead, the late senescence phenotype was mapped to two genomic loci. The *old103* gene was mapped to the same region as the *old101* gene (data not shown) and therefore crossed to *old101*. The F1 progeny showed a late senescence phenotype (Table 1). Since both *old101* and

old103 segregate as recessive monogenic traits this demonstrates that these mutations are allelic. *old101* was renamed as *old101-1* and *old103* as *old101-2*. Further mapping showed that the *old101* mutations map to a single locus on chromosome 5 (data not shown). Similarly, the *old104* and *old106*

mutations segregated as monogenic traits in the *Ler-0* background, but mapped to two different loci after a cross with the Col-0 accession. The results show that we have successfully isolated several independent stay-green loci.

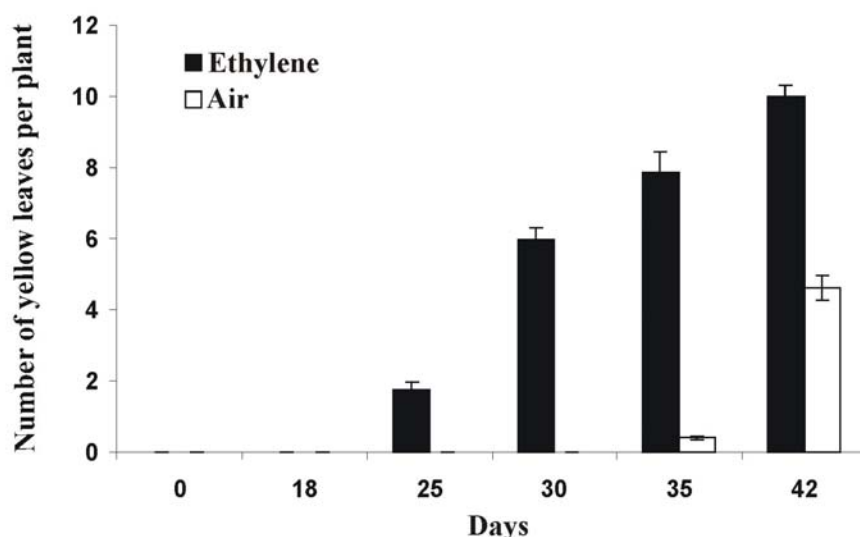


Figure 1 The effect of ethylene treatment on visible leaf yellowing of wild type plants. *Ler-0* plants were grown either continuously in air for the indicated number of days or first in air for up to 4 days before the indicated days, followed by 3 days growth in air supplemented with ethylene and one additional day of growth in air. The number of yellow leaves, including cotyledons, was subsequently counted as described in the methods section. Data are shown as mean $\pm$ standard deviation of at least 25 plants.

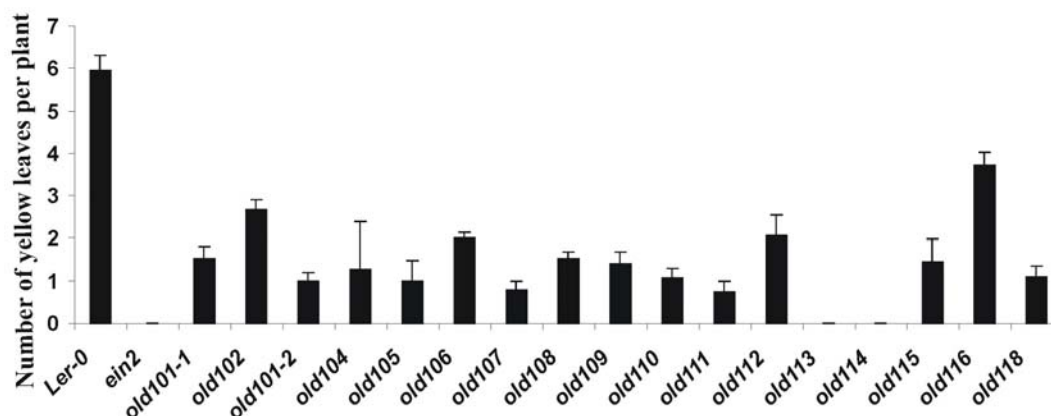


Figure 2 The effect of ethylene treatment on visible leaf yellowing of *old* mutants. Plants were grown in air for 26 days, followed by 3 days of growth in air supplemented with ethylene and one additional day of growth in air. The number of yellow leaves, including cotyledons, was subsequently counted. Data are shown as mean $\pm$ standard deviation of at least 25 plants.

Table 1 Genetic segregation analysis of late senescence mutants in F1 and F2 generations.

Male	Female	Generation	Wild type	Mutant	Genetic behaviour	χ^2	P	Genome location
<i>old101-1</i>	<i>Ler-0</i>	F1	51	0	Recessive			
		F2	123	54	1:3	2.86	0.09	Chr V
<i>old101-2</i>	<i>Ler-0</i>	F1	32	0				
<i>old101-1 old101-2</i>		F1	0	27				
<i>old102</i>	<i>Ler-0</i>	F1	51	0	Recessive			
		F2	135	33	1:3	2.44	0.19	Chr I
<i>old104</i>	<i>Ler-0</i>	F1	68	0	Recessive			
		F2	132	38	1:3	0.2	0.65	Chr III and IV
<i>old105</i>	<i>Ler-0</i>	F1	12	8	Co-dominant			
<i>old106</i>	<i>Ler-0</i>	F1	68	0	Recessive			
		F2	100	36	1:3	1.44	0.23	Chr I and IV
<i>old107</i>	<i>Ler-0</i>	F1	13	7	Co-dominant			
<i>old108</i>	<i>Ler-0</i>	F1	30	0	Recessive			
<i>old109</i>	<i>Ler-0</i>	F1	0	15	Dominant			
<i>old110</i>	<i>Ler-0</i>	F1	30	0	Recessive			
<i>old111</i>	<i>Ler-0</i>	F1	9	6	Co-dominant			
<i>old112</i>	<i>Ler-0</i>	F1	30	0	Recessive			
<i>old115</i>	<i>Ler-0</i>	F1	0	29	Dominant			
<i>old116</i>	<i>Ler-0</i>	F1	30	0	Recessive			
<i>old118</i>	<i>Ler-0</i>	F1	16	9	Co-dominant			

The old plants (Male) were crossed to *Ler-0* wild type plants (Female) and the indicated number of F1 plants was investigated to resemble either the wild type or the mutant phenotypes. For several mutants the F2 generation was scored for segregation of the late senescence phenotype after ethylene treatment. The χ^2 standard test was applied to determine the segregation pattern of several old genes. For mapping the old mutants (male) were crossed to Col-0 wild type plants (female) as described in the methods section.

Ethylene responses of late senescence mutants

The late senescence mutants (16 in total) were isolated based on the senescence phenotype after ethylene exposure. Thus the phenotype of these mutants could be a result of a reduced sensitivity to the hormone ethylene. Therefore we tested another ethylene response during early seedling development. Wild type seedlings that are grown on plates containing the precursor of ethylene, ACC, show a so-called triple-response (Neljubow 1901). We tested the response of the *old* mutants to ACC and compared it to that of the wild type *Ler-0*.

The wild type and *old* mutant seedlings were grown on Murashige and Skoog (MS) medium with and without 10 μ M ACC for 5 days in darkness and subsequently the hypocotyl length was measured.

Table 2 shows that ACC treatment of wild type seedlings caused a reduction in hypocotyl extension to approximately 40% of those germinated on media without ACC. The mutant seedlings can be categorised into two groups, based on their response to the ACC treatment. The first group responded strongly to the ACC treatment, in a similar way as the wild type (i.e., ACC treatment resulted in a more than 50% reduction of

Table 2 The hypocotyl length of wild type and mutant seedlings grown on Murashige and Skoog medium with and without 10 μ M ACC.

Plant	0 μ M ACC (mm)	10 μ M ACC (mm)	Ratio
<i>Ler-0</i>	13.4 $\pm$ 0.2	5.1 $\pm$ 0.2	0.38
<i>ein2</i>	14.4 $\pm$ 0.4	15.3 $\pm$ 0.4	1.06
<i>old101-1</i>	11.3 $\pm$ 0.2	5.0 $\pm$ 0.2	0.44
<i>old101-2</i>	11.5 $\pm$ 0.3	4.8 $\pm$ 0.4	0.41
<i>old102</i>	13.4 $\pm$ 0.4	4.4 $\pm$ 0.2	0.33
<i>old104</i>	12.0 $\pm$ 0.6	4.8 $\pm$ 0.2	0.40
<i>old105</i>	12.7 $\pm$ 0.3	5.3 $\pm$ 0.3	0.42
<i>old106</i>	7.2 $\pm$ 0.3	5.1 $\pm$ 0.2	0.70
<i>old107</i>	11.3 $\pm$ 0.4	4.0 $\pm$ 0.2	0.36
<i>old108</i>	11.1 $\pm$ 0.9	4.9 $\pm$ 0.2	0.44
<i>old109</i>	12.2 $\pm$ 0.2	7.1 $\pm$ 0.9	0.58
<i>old110</i>	11.5 $\pm$ 0.5	4.8 $\pm$ 0.2	0.42
<i>old111</i>	10.6 $\pm$ 0.2	4.0 $\pm$ 0.2	0.38
<i>old112</i>	9.1 $\pm$ 0.6	4.1 $\pm$ 0.1	0.45
<i>old113</i>	13.5 $\pm$ 0.5	10.0 $\pm$ 0.6	0.74
<i>old114</i>	10.1 $\pm$ 0.4	7.4 $\pm$ 0.4	0.74
<i>old115</i>	10.8 $\pm$ 0.5	8.9 $\pm$ 0.7	0.82
<i>old116</i>	7.4 $\pm$ 0.6	3.8 $\pm$ 0.3	0.52
<i>old118</i>	13.0 $\pm$ 0.5	6.0 $\pm$ 0.5	0.46

Data represents the average of 25 seedlings $\pm$ SD. Ratio, hypocotyl length when grown without ACC over growth on medium containing ACC; *ein2*, *ethylene-insensitive mutant2* (Guzman & Ecker 1990).

hypocotyl extension) and include the *old101* alleles, *old102*, *old104*, *old105*, *old107*, *old108*, *old110*, *old111*, *old112* and *old118*. The *old116* mutant also responds strongly to the ACC treatment, but has relatively short hypocotyls when grown without ACC. The late ethylene-induced senescence phenotype of these *old* mutants, therefore, is not a result of a mutation in the ethylene-signalling pathway.

The second group of mutants consisting of *old106*, *old109*, *old113*, *old114*, *old115* and *old116* responded less to the ACC treatment. Thus the late senescence phenotype of the second group of mutants may, partially, be a result of reduced ethylene signalling. Further research should determine whether one or more of these *old* mutants are allelic to known ethylene-signalling mutants such as the *ein2* mutant.

Phenotypic characterisation of the stay-green mutants

The *old* mutations may affect other aspects of plant growth apart from the late

senescence phenotype. Thus the *old* mutants were observed throughout development and phenotypic particularities are summarised in Table 3 and representative 30-day-old ethylene treated wild type and *old* plants are shown (Figure 3). All mutants have fewer yellow leaves than the wild type but other phenotypes were observed as well. The most conspicuous phenotype is the flowering time phenotype. Most mutants have a slightly late or late flowering phenotype. Interestingly, the *old102* mutant has an early flowering phenotype. Furthermore, some *old* mutants appear to have a leaf phenotype (Table 3). Some late senescence mutants have smaller leaves and/or petioles, while others have bigger leaves or longer petioles. The meristem of *old112* appears to develop faster, resulting in more leaves. There does not seem to be a correlation between the senescence phenotype and the leaf and/or development phenotype.

The results suggest there is a relation between senescence and flowering time. The mutants, furthermore, have a variety of

pleiotropic phenotypes. However, most of the mutants were not backcrossed twice and thus still carry some mutagenesis load.

Further backcrosses should reveal whether all the pleiotropic phenotypes are linked to the senescence phenotypes.

Table 3 Phenotypic features of 30-old-day late senescence mutants after ethylene treatment, as compared to the wild type Ler-0.

Mutant	Flowering phenotype	Leaf phenotype	Number of rosette leaves ¹	Number of backcrosses ²
WT (Ler-0)	Normal	Wild type	10 leaves	Wild type
<i>old101</i>	Normal	Darkened	10 leaves	Twice
<i>old102</i>	Early	Smaller	6 leaves	Twice
<i>old104</i>	Normal	Wild type	10 leaves	None
<i>old105</i>	Late flowering	Darkened, smooth margin	12 Leaves	Once
<i>old106</i>	Normal	Bigger, wider and protruded lamina	6 leaves	Once
<i>old107</i>	Normal	Narrow, longer petiole, elongated lamina	10 leaves	Once
<i>old108</i>	Late flowering	Smaller, rounded and wider lamina	12 leaves	Once
<i>old109</i>	Late flowering	Bigger	12 Leaves	Once
<i>old110</i>	Normal	Wild type	8 leaves	None
<i>old111</i>	Normal	Smaller, narrow lamina, longer petiole	9 leaves	None
<i>old112</i>	Late flowering	Smaller, rounded and darkened lamina	14 leaves	None
<i>old113</i>	Late flowering	Rounded lamina, longer petiole	8 leaves	None
<i>old114</i>	Late flowering	Wild type	10 leaves	Once
<i>old115</i>	Late flowering	Wild type	10 leaves	Once
<i>old116</i> ³	Late flowering	Smaller, darkened, rounded and wider lamina	10 leaves	None
<i>old118</i>	Late flowering	Narrow lamina, long petiole	8 leaves	None

¹The number of rosette leaves only, not including cauline leaves.

²The phenotype was observed in mutants that were not backcrossed (none) or backcrossed once or twice.

³59-day-old mutant plants grown in air show more signs of yellowing in rosette leaves as compared with wild type plants.

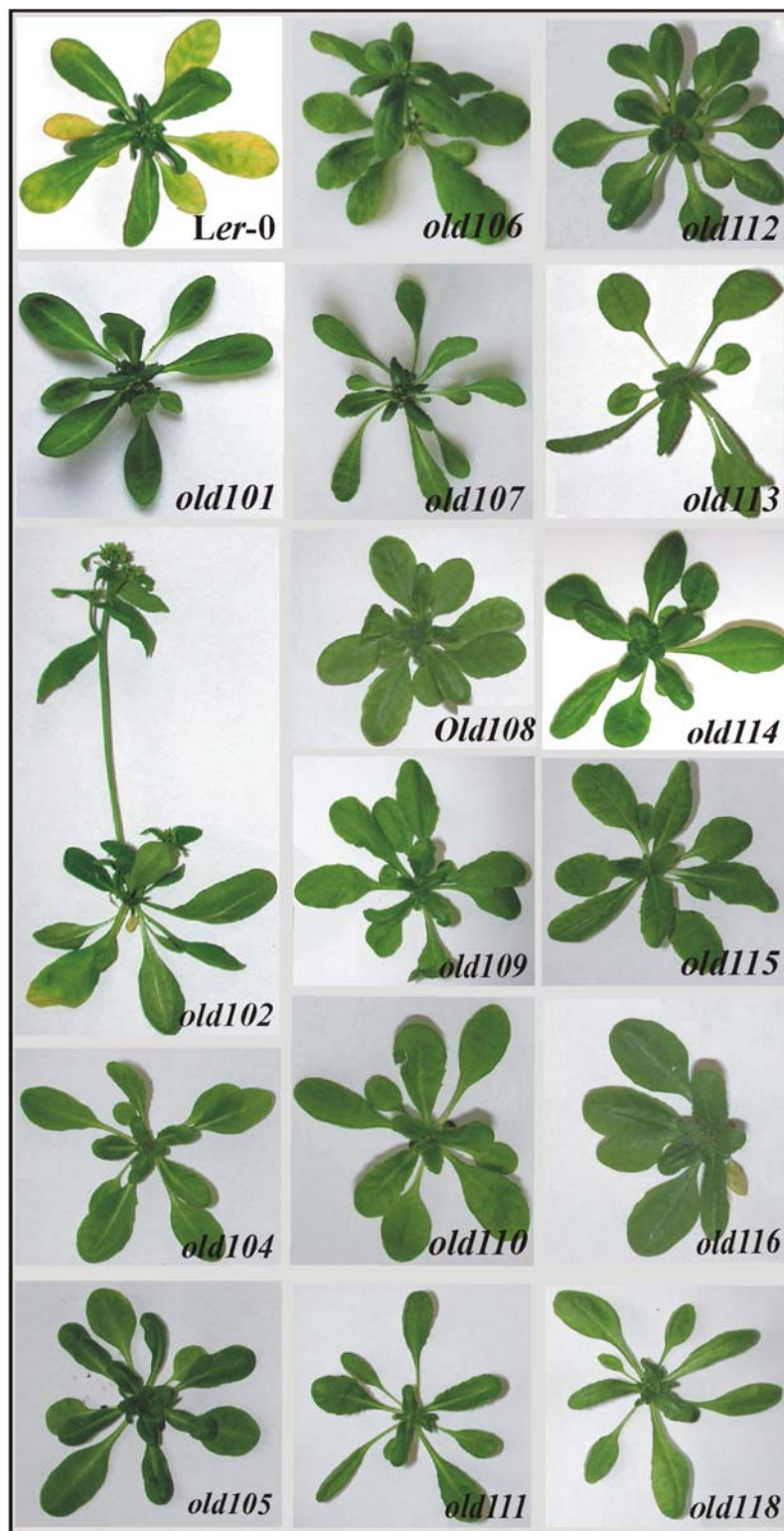


Figure 3 Photographs of representative 30-day-old wild type and *old* mutants. The plants were grown for 26 days in air, followed by 3 days of growth in air supplemented with ethylene and one additional day of growth in air.

Discussion

Leaf senescence marks the final developmental stage of a leaf and functions both as a detrimental process, resulting in leaf death, and a beneficial process as nutrients recovered from the dying leaf are redistributed to growing parts of the plant. Therefore it is not surprising that senescence can be delayed or accelerated by many developmental and environmental cues (Lim *et al.* 2007; Schippers *et al.* 2007).

In efforts aimed at identifying regulatory components of senescence, genetic strategies have been highly effective, certainly in *Arabidopsis* (Lim *et al.* 2007). Because of the well-documented role of ethylene we aimed at identifying genes that, when mutated, cause a stay-green phenotype after ethylene treatment. Here we present the identification of 16 mutants with a stay-green phenotype. To find out whether the stay-green phenotype after ethylene treatment is a result of insensitivity to ethylene or not, we took advantage of the triple response test to check responses of late senescence mutant seedlings to ethylene on plates containing the precursor of ethylene, ACC. The results of the experiment put late senescence mutants in two groups: mutants that respond to ethylene in the same way as the wild type (*old101* alleles, *old102*, *old104*, *old105*, *old107*, *old108*, *old110*, *old111*, *old112* and *old118*) or mutants that show a reduced response to the treatment (*old106*, *old109*, *old113*, *old114*, *old115* and *old116*). However, it should be noted that, in comparison with the wild type, the *old106* and *old116* mutants had a strongly reduced hypocotyl length in the absence of ACC, and thus limiting the maximum possible effect of the ACC treatment. Nevertheless, the *old* mutants still responded to the ethylene treatment to some extent and further research is required to confirm or reject the possibility that the stay-green phenotype in the *old* mutants is the result of a reduced ethylene response. The other mutants showed a wild type response to the ACC

treatment in seedlings and this suggests that the ethylene signal transduction pathway is fully functional. It may be that these mutants have a lesion in senescence induction, specifically after ethylene treatment.

Genetic characterisation of four of the stay-green mutants (*old101* alleles, *old102*, *old104*, *old106*) resulted in the identification of at least four loci that are involved in controlling the leaf senescence process. Thus multiple genetic loci are required to control the leaf senescence process. Several independent genetic loci involved in the regulation of leaf senescence have been reported (Kim *et al.* 2007). In addition, a number of *Arabidopsis* QTL that are responsible for variation in leaf and plant longevity were identified (Levey & Wingler 2005; Luquez *et al.* 2006) and suggests that leaf longevity is regulated by a wide range of genes influencing different molecular pathways in *Arabidopsis*. The *old* mutants isolated here are healthy and look very similar to the wild type under standard growth conditions. However, various pleiotropic phenotypes were found in the mutants. In several cases leaf morphology differences were found. The significance of these phenotypes is unclear. Leaf phenotypes frequently occur and Berna *et al.* (1999) identified 853 leaf morphology mutants that yielded viable M3 inbred progeny. Most of the stay-green mutants have not been backcrossed or were backcrossed only once and therefore still carry a resident EMS mutation load. Further genetic and phenotypic analysis is required to determine whether the leaf phenotypes co-segregate with the stay-green phenotype. One conspicuous phenotype seems to correlate with the stay-green phenotype. Most of the mutants were delayed in flowering and this confirms earlier observations (Levey & Wingler 2005; Barth *et al.* 2006; Wu *et al.* 2008).

In conclusion, we have successfully isolated 16 *Arabidopsis* mutants that stay-green after ethylene treatment indicating that the

selected *old* mutants prolonged leaf longevity. Future study will be aimed at identification and subsequent functional analysis of the *old* stay-green mutants and will help to reveal the mechanism of the regulation of senescence in *Arabidopsis*. Leaf senescence can both positively and negatively influence harvest yield and quality (Yang and Zhang, 2006; Guo and Gan, 2007). An increased understanding of *OLD* genes will provide useful information for developing new genetic strategies toward extending the shelf life and improving economically important traits of crop plants.

Acknowledgements

We would like to thank Bert Venema for his excellent help in the greenhouse. This work was supported in part by a grant from the Ministry of Science, Research and Technology of the Islamic Republic of Iran to Reza Shirzadian-Khorramabad.

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